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Carboxyl-terminal domain of the Epstein–Barr virus nuclear antigen is highly immunogenic in man

(*Escherichia coli* expression plasmid/monoclonal antibodies/quantitation of serum antibody levels/ELISA)

GREGORY MILMAN*, ALAN L. SCOTT*, MYUNG-SAM CHO†, STEVEN C. HARTMAN*, DAVID K. ADES*, GARY S. HAYWARD†, PING-F. KI†, J. THOMAS AUGUST†, AND S. DIANE HAYWARD†

*Department of Biochemistry, School of Hygiene and Public Health, and †Department of Pharmacology and Experimental Therapeutics, School of Medicine, The Johns Hopkins University, Baltimore, MD 21205

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ABSTRACT **The carboxyl-terminal one-third of the Epstein–Barr virus nuclear antigen (EBNA-1) encoded by the *Bam*HI restriction fragment K was synthesized in *Escherichia coli* by use of a high-expression plasmid. The resultant 28-kDa EBNA fusion polypeptide, comprising 5–10% of the total soluble bacterial protein, was purified to apparent homogeneity by phosphocellulose and hydroxylapatite column chromatography. Both rabbit monospecific antibodies and mouse monoclonal antibodies against 28-kDa EBNA gave nuclear immunofluorescence staining on Epstein–Barr virus (EBV)-infected lymphoblastoid cell lines and recognized the appropriate intact EBNA polypeptide bands on immunoblots. An ELISA with the purified 28-kDa EBNA as antigen was used to quantitate anti-EBNA antibody in human serum samples. The ELISA method was ≈100-fold more sensitive than the classical anticomplement immunofluorescence assay. Anti-EBNA antibody was detected in sera from 100% of normal individuals who were seropositive for the viral capsid antigen, and low anti-EBNA titers were detected in serum from most patients with acute infectious mononucleosis. The assay gave the expected pattern of titers in sera from patients with rheumatoid arthritis, Burkitt lymphoma, or nasopharyngeal carcinoma, thus confirming the validity of this purified reagent for assessing EBNA antibody status. Approximately 10% of normal individuals and rheumatoid arthritis patients had anti-EBNA titers as high as those seen in nasopharyngeal carcinoma patients. In these high-titer individuals, >1% of the total IgG are antibodies that recognize 28-kDa EBNA, which indicates that the carboxyl-terminal domain of EBNA is highly immunogenic.**

Epstein–Barr virus (EBV) is one of the most common viruses infecting man, and antibodies to EBV proteins are present in >80% of human serum samples. EBV is the etiological agent of infectious mononucleosis and has been implicated in the pathogenesis of Burkitt lymphoma and nasopharyngeal carcinoma. The 172,000-base-pair (bp) DNA genome of EBV is found in these tumors and in all "immortalized" permanent B-cell lymphoblast lines as multicopy circular plasmids or episomes. The immune response of patients with EBV-related diseases can be an important indicator for clinical evaluation and treatment. For example, the presence of IgA antibodies to the viral capsid antigen is virtually diagnostic for undifferentiated nasopharyngeal carcinoma (1–4), and the antibody response to the early-antigen complex forms a prognostic indicator of the outcome of this disease (5). We anticipate that the value of such monitoring would be enhanced if assays based on total infected cell extracts could be complemented by quantitative ELISAs measuring the antibody response to individual viral proteins.

All cells carrying EBV DNA, including the tumor cells in Burkitt lymphoma and nasopharyngeal carcinoma, express the nuclear antigen EBNA. Two separate components of EBNA have been identified by serology (6, 7). The EBNA-1 polypeptide was shown by DNA transfection experiments to be synthesized from the *Bam*HI restriction fragment K (8, 9). Within *Bam*HI K lies a 2.0-kilobase (kb) open reading frame, 700 bp of which is composed exclusively of three repeated triplet sequences, GGA, GGG, and GCA (10, 11). The size of the repeat array varies among different EBV isolates and the EBNA polypeptide shows corresponding size variations ranging from 68 kDa to 84 kDa (12–14). Hennessy and Kieff (6) used rabbit antiserum raised against a bacterially synthesized product of the repeat array to demonstrate that these sequences are translated in the EBNA polypeptide as a stretch of repeated glycine and alanine residues. Both this antiserum and one produced against a synthetic peptide containing -Gly-Gly-Ala- (15) recognized EBNA in immunofluorescence assays and in immunoblot analyses. However, the repeat DNA sequence has homology to cellular DNA (16), and antisera to the -Gly-Gly-Ala- peptides also reacted with cellular proteins (15, 17). The EBNA protein is known to have DNA-binding properties and to be required in *trans* for maintenance of the multicopy circular state of transfected DNA containing the EBV plasmid replication origin region (18).

Waldman *et al.* (19) described the use of high-expression plasmids to synthesize large quantities of herpes simplex virus thymidine kinase in bacteria. In this report, we describe the bacterial synthesis of a 28-kDa fusion polypeptide that contains the carboxyl-terminal fragment of EBNA and lacks the Gly-Gly-Ala repeat region. This 28-kDa EBNA, which can be obtained in large quantity and is easily purified, was used to generate anti-EBNA monospecific and monoclonal antibodies and to develop a sensitive ELISA for quantitating anti-EBNA antibodies in human serum.

MATERIALS AND METHODS

Cell Lines. Mouse fibroblast cell lines synthesizing EBNA (LEK cells) were established by transfecting LTK^- cells with the EBV (P3HR-1) *Bam*HI K fragment inserted into pSV2neo (20) and selecting for G418 (geneticin) resistance and EBNA expression. LTK^- cells and LEK cells were grown in Dulbecco's modified Eagle's medium, and the lymphoblastoid lines Raji, P3HR-1, and BJAB were grown in RPMI 1640. All media were supplemented with 10% fetal calf serum (Hyclone, Sterile Systems, Logan, UT).

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Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV-encoded nuclear antigen; ACIF, anticomplement immunofluorescence; kb, kilobase(s); bp, base pair(s).

Plasmid Construction. The high-expression plasmid pHE6 (Fig. 1) contains essential regions of the plasmid pGM10 (19) inserted into a derivative of the plasmid pUC8 (21). Specifically, the *Hae* II–*Sma* I region of pUC8 was replaced with two adjacent segments of λ phage DNA (22): that from the *Hae* II site at base 37,061 to the *Bgl* II site at base 38,103 and that from the *Bgl* II site at base 35,711 to the *Hpa* I site at base 35,261. In addition, a synthetic DNA fragment (CCGGATCCCC) was inserted between the *Hpa* I site and the GGG of the *Sma* I site of pUC8. Foreign DNA may be inserted into the unique *Bam*HI, *Sma* I, *Sal* I, and *Pst* I restriction sites. Plasmid pHE6 produces polypeptides from inserted foreign DNA as "run-on" proteins of the λ phage N protein. The synthesis of these proteins is regulated by the λ temperature-sensitive repressor. A 2236-bp *Sma* I subfragment of EBV (P3HR-1) *Bam*HI fragment K was inserted at the *Sma* I site of pHE6 in both the sense and the antisense directions to produce plasmids pNAK28 and pNAK28$^-$ (Fig. 1).

Purification of Bacterially Synthesized 28-kDa EBNA. *Escherichia coli* K-12 DH1 containing pNAK28 were induced to synthesize the 28-kDa EBNA by a shift in temperature from 30°C to 42°C as described (19). The bacteria were harvested by centrifugation, washed, resuspended in 1/25th the original volume with 50 mM Tris Cl, pH 7.5/10 mM EDTA/1 mM phenylmethylsulfonyl fluoride (PMSF), and frozen. The frozen bacteria were thawed in an equal volume of 50 mM Tris HCl, pH 7.5/10 mM EDTA/1 mM PMSF/lysozyme (0.5 mg/ml) and placed on ice for 15 min. Nonidet P-40 (Sigma) was added to a final concentration of 0.1% and the mixture was kept on ice for 15 min. The solution was mixed with 0.2 volume of 5.0 M NaCl, and cell debris was removed by centrifugation for 1 hr at 27,000 × *g* in a Sorvall SS-34 rotor. The supernatant was diluted 4-fold with 50 mM Tris Cl, pH 7.5/1 mM PMSF and applied to a phosphocellulose column (Whatman P-11) equilibrated in 50 mM Tris Cl, pH 7.5/200 mM NaCl/1 mM PMSF. The column was washed in the same buffer and the 28-kDa EBNA protein was eluted with a linear gradient from 200 to 600 mM NaCl in 50 mM Tris Cl, pH 7.5/1 mM PMSF. Fractions that contained 28-kDa EBNA on analysis by NaDodSO$_4$/PAGE were pooled, diluted with an equal volume of water and applied to a hydroxylapatite column (Bio-Gel HTP) equilibrated in 50 mM Tris Cl, pH 7.5/250 mM NaCl. The column was washed with the same buffer and 28-kDa EBNA was eluted with a linear gradient of 0–250 mM sodium phosphate (pH 7.5) in 250 mM NaCl/50 mM Tris Cl, pH 7.5.

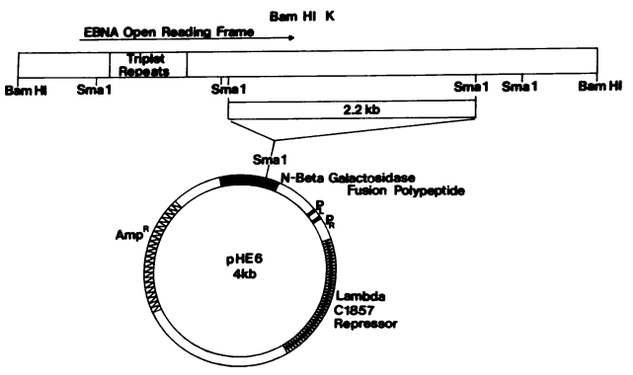

FIG. 1. Derivation of the pNAK28 plasmids encoding 28-kDa EBNA. The diagram illustrates the size and structure of the pHE6 expression plasmid and of the EBV *Bam*HI K fragment. The pHE6 plasmid contains the bacteriophage λ strong leftward and rightward promoters (P_L and P_R), the λ temperature-sensitive repressor gene (CI857), and codons for the amino-terminal 33 amino acids of the λ N protein inserted into plasmid pUC8. The 2.2-kb *Sma* I subfragment of the EBV *Bam*HI K fragment, which maps to the right of the triplet repeat region and contains the carboxyl-terminal third of the EBNA open reading frame, was ligated in both orientations into the single *Sma* I site of pHE6. Amp^R, ampicillin-resistance gene.

Antibody Preparation. New Zealand White rabbits were injected subcutaneously with 300 μg of purified 28-kDa EBNA suspended in Freund's complete adjuvant and given booster injections at 10- to 14-day intervals with 300 μg of 28-kDa EBNA suspended in incomplete adjuvant. Monoclonal antibodies to 28-kDa EBNA were prepared by methods previously described (23). Briefly, spleen cells from BALB/c mice immunized with 28-kDa EBNA were fused with P3-X63Ag8 myeloma cells. Culture supernatants were screened for antibody capable of immunoprecipitating 28-kDa EBNA, and cells from positive wells were cloned twice in soft agar. The five monoclonal antibodies used in this work (EBNA-2D1, EBNA-3C6, EBNA-6A1, EBNA-6A3, and EBNA-6B1) were all of the IgG1 class and were prepared as ascites fluid.

Immunological Assays for EBNA. Immunological reagents were purchased from Cappel Laboratories (Cochranville, PA) and Bethesda Research Laboratories (Bethesda, MD). For immunofluorescence assays, the cells were fixed in methanol and then incubated with rabbit serum and stained by anticomplement immunofluorescence (ACIF) (24) or with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG. Slides incubated with monoclonal antibodies were stained using biotin-tagged anti-mouse IgG followed by FITC-conjugated streptavidin. Immunoblot analyses were carried out by published procedures (17, 25). The protein blots were incubated with human, rabbit, or monoclonal serum for 90 min at room temperature, washed, and reacted with the appropriate alkaline phosphatase-tagged anti-IgG antibody. Bands were visualized by incubation with color-producing substrate (napthol AS-BI, fast blue, and fast violet).

ELISA for Anti-EBNA Antibody. Microtiter plates coated with purified 28-kDa EBNA (40 ng per well in 0.5 M sodium carbonate buffer at pH 9.6) were incubated first with blocking solution (2% normal goat serum in 0.15 M NaCl/0.1 M sodium phosphate, pH 7.4/0.05% Tween 20) and then with patient serum diluted in blocking solution. After extensive washing, specific binding was detected by use of peroxidase-conjugated goat anti-human IgG. The peroxidase-tagged antibody was detected by 15-min incubation in color-producing substrate (*o*-phenylenediamine). The titer was defined as that dilution which gave an absorption of 0.75 at 492 nm, as measured with a Flow Laboratories Titertek Multiscan MC. The concentration of anti-28-kDa EBNA IgG in serum was determined by reference to a standard curve.

RESULTS

Synthesis and Purification of 28-kDa EBNA. We chose to express the carboxyl-terminal one-third of *Bam*HI fragment K-encoded EBNA to avoid the complications of including the (Gly-Gly-Ala)$_n$ region and to determine whether other portions of the protein were also immunogenic. A 2.2-kb *Sma* I subfragment from *Bam*HI fragment K was inserted in phase behind the 5′ portion of phage λ N protein in the expression plasmid pHE6 (Fig. 1). This subfragment contains that portion of the EBNA coding region mapping between nucleotides 109,298 and 109,872 in the EBV (B95-8) DNA sequence (11). The construct pNAK28 should direct the synthesis of a fusion protein containing 36 amino acids from the bacteriophage λ N protein and linker and 191 amino acids from the carboxyl terminus of EBNA. The predicted size of the fusion protein is 28 kDa. Inactivation of the temperature-sensitive λ repressor in bacteria containing pNAK28 induced the synthesis of a 28-kDa fusion polypeptide which was the dominant band detected by Coomassie blue staining after NaDodSO$_4$/PAGE of the crude bacterial extracts (Fig. 2, lane B). This protein was not present in extracts of bacteria containing the plasmid with the EBNA insert in the antisense

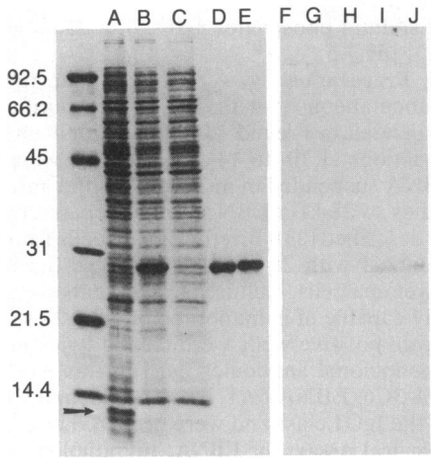

FIG. 2. Synthesis and purification of the 28-kDa EBNA fusion polypeptide. Lanes A–E contain samples of *E. coli* protein extracts and of purified 28-kDa EBNA fractions separated by $NaDodSO_4$/PAGE and stained with Coomassie blue. The immunoblots of lanes A–E are shown in lanes F–J. The blot was reacted with a 1:1000 dilution of serum from a nasopharyngeal carcinoma patient. Size markers are located in the left lane (values in kDa at left). Lanes A and F: crude extract (16 μg of protein) of cells containing pNAK28 with the EBNA insert in the antisense orientation. Arrow shows the location of the presumed antisense peptide fragment. Lanes B and G: crude extract (13 μg) of cells containing pNAK28 with the EBNA insert in the sense orientation. Lanes C and H: flow-through polypeptides (10 μg) which did not adsorb to phosphocellulose. Lanes D and I: eluted and pooled phosphocellulose fractions (1.3 μg) containing 28-kDa EBNA. Lanes E and J: eluted and pooled hydroxylapatite fractions (1.3 μg) containing 28-kDa EBNA.

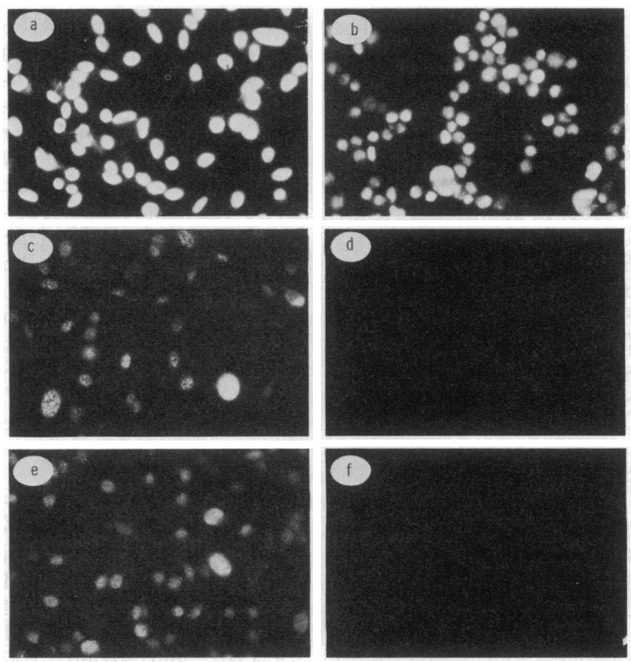

FIG. 3. Detection of EBNA by immunofluorescence using rabbit serum and monoclonal antibodies raised against the bacterially synthesized 28-kDa EBNA. LEK fibroblasts (*a* and *c*), P3HR-1 lymphoblastoid cells (*b*), or LTK^- fibroblasts (*d*) were incubated with rabbit serum and stained by ACIF (*a*, *b*, and *d*) or indirect immunofluorescence (*c*). LEK (*e*) or LTK^- cells (*f*) were incubated with a mixture of five monoclonal antibodies and stained by a biotin–streptavidin indirect immunofluorescence reaction. (×1000.)

orientation (lane A) or in extracts of bacteria containing the pHE6 expression vehicle only (not shown). When pNAK28 extracts were applied to phosphocellulose, most of the protein passed through the column (lane C), but the 28-kDa polypeptide bound and was eluted between 0.5 and 0.6 M NaCl (lane D). Minor contaminants were removed by chromatography on hydroxylapatite (lane E), where the 28-kDa polypeptide was eluted between 120 mM and 160 mM phosphate. This 28-kDa fusion fragment of EBNA comprised ≈8% of the soluble protein in extracts of induced bacteria containing pNAK28 and it was the only band recognized by EBNA-positive human serum in immunoblots of the bacterial extracts (lanes F–J), thus confirming that it represented the product of the inserted EBNA coding region. The purified 28-kDa EBNA polypeptide was used to elicit monospecific and monoclonal antibodies in rabbits and mice, respectively.

Correct Specificity of Rabbit Antiserum and Monoclonal Antibodies Raised Against the 28-kDa EBNA Polypeptide. The reactivity of anti-28-kDa EBNA antisera in indirect immunofluorescence assays is illustrated in Fig. 3. Positive nuclear fluorescence was detected in EBV-carrying lymphoblastoid cell lines (Fig. 3*b*) and in the DNA-transfected fibroblast cell line (LEK) expressing EBNA (Fig. 3 *a*, *c*, and *e*), but not in the EBV genome-negative BJAB lymphoblastoid cell line or in the mouse LTK^- line from which the LEK transfectants were derived (Fig. 3 *d* and *f*). On LEK cells, EBNA was detectable with the rabbit serum by either the ACIF amplification procedure (Fig. 3*a*) or more directly with FITC-conjugated anti-rabbit IgG (Fig. 3*c*). EBNA was barely detectable by immunofluorescence using individual monoclonal antibodies. However, a pool of five monoclonal antibodies gave positive results in a biotin–streptavidin-based indirect immunofluorescence assay with either EBV-carrying lymphoblastoid cell lines (results not shown) or LEK fibroblasts (Fig. 3*e*). The enhanced immunofluorescence obtained by pooling the monoclonal antibodies suggests that the individual antibodies are probably directed against different epitopes.

To demonstrate that the antibodies raised against the 28-kDa EBNA recognized the appropriate intact EBNA polypeptides, we carried out immunoblotting experiments. As shown in Fig. 4, the rabbit antiserum, individual monoclonal antibodies, and an EBNA-positive human control

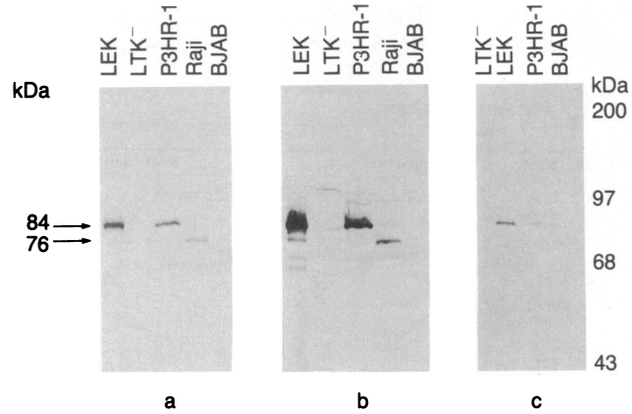

FIG. 4. Detection of EBNA by an immunoblot assay using human or rabbit serum and mouse monoclonal antibodies. Extracts of EBNA-positive (LEK, P3HR-1, and Raji) and EBNA-negative (LTK^- and BJAB) cell lines were electrophoresed in a $NaDodSO_4$/12.5% polyacrylamide gel and transferred to a nitrocellulose membrane (25). The protein blots were incubated with dilutions of EBNA-positive, nasopharyngeal carcinoma patient serum (*a*), rabbit hyperimmune serum (*b*), or the EBNA-6A3 monoclonal antibody (*c*) and then reacted with the appropriate alkaline phosphatase-conjugated second antibody. The apparent sizes of the EBNA polypeptides in our gel system (84 and 76 kDa) are somewhat larger than those reported by other investigators (12–14).

serum all recognized a polypeptide of apparent molecular size 84 kDa in extracts from P3HR-1 lymphoblasts and in extracts from LEK cells (converted by transfection with the P3HR-1 *Bam*HI K fragment). This 84-kDa band was not present in EBV-negative BJAB lymphoblasts or in LTK$^-$ cells. The Raji EBNA polypeptide is smaller than that of P3HR-1 (12) and as expected, the rabbit antiserum and the human EBNA-positive control serum both immunostained an appropriately smaller protein in Raji cell extracts.

Sensitive ELISA for Examining the Spectrum of Anti-EBNA Antibody in Human Serum. The availability of large quantities of pure 28-kDa EBNA antigen made it feasible to develop a rapid and highly specific ELISA for quantitation of antibody titers in clinical samples. The assay depended upon the carboxyl-terminal portion of EBNA and not just the Gly-Gly-Ala repeats being immunogenic. A summary of ELISA determinations of anti-28-kDa EBNA IgG in 222 serum samples from normal adults or patients with infectious mononucleosis, rheumatoid arthritis, Burkitt lymphoma, or nasopharyngeal carcinoma is presented in Fig. 5. All normal individuals (88/88) who were EBV-seropositive (i.e., whose serum contained antibodies to the EBV viral capsid antigen as measured by indirect immunofluorescence on the virus-producing lymphoblast cell line B95-8) had detectable levels of anti-EBNA antibody (titers greater than 1:40) and none

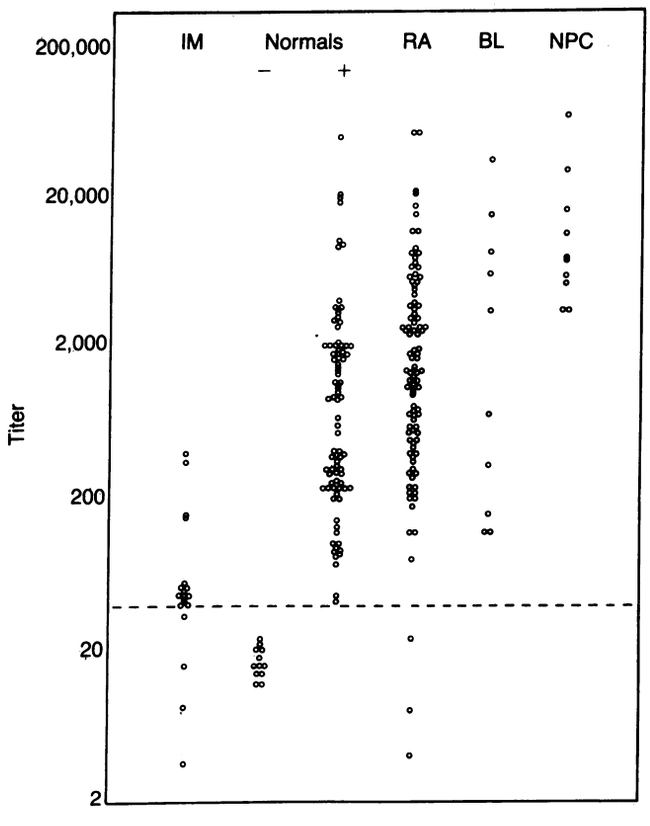

FIG. 5. Anti-28-kDa EBNA titers of patient sera as measured by an ELISA using bacterially synthesized 28-kDa antigen. The ELISA titer was defined as that serum dilution which gave an absorbance of 0.75 at 492 nm. The number of samples tested were as follows: infectious mononucleosis (IM), 19 (all samples were anti-IgM viral capsid antigen-positive with titers between 1:20 and 1:160); rheumatoid arthritis (RA), 83; Burkitt lymphoma (BL), 10; and nasopharyngeal carcinoma (NPC), 10. The normal group (ages early twenties to late thirties) was divided into those individuals who were seropositive (+) for the EBV viral capsid antigen (88 samples) and those who were negative (−) for this antigen (12 samples). The broken line marks the division between EBNA-positive and EBNA-negative.

(0/12) of the seronegative normal samples gave positive ELISA reactions (titers equal to or less than 1:20). ELISA dilution titers for the seropositive normal individuals covered a range from 1:40 to 1:50,000, with a median titer of 1:1000. All Burkitt lymphoma and nasopharyngeal carcinoma sera tested and 80/83 of the rheumatoid arthritis sera were positive. The titers of the seropositive rheumatoid arthritis and Burkitt lymphoma patients spanned the same range as the normals, but the median titers of these patients were 1:1400 and 1:2400, somewhat higher than that of the normal group. The 10 nasopharyngeal carcinoma sera all showed elevated EBNA titers. The median titer was 1:8000 and 80% of the samples had titers greater than 1:5000, in contrast to the normal group where only 8% of the samples fell into this category. Although the infectious mononucleosis sera were EBNA-negative by ACIF, 15 of the 19 samples had low but demonstrable anti-EBNA antibody titers by ELISA. The median titer for these sera was 1:50. In general, an ELISA titer of 1:500 was approximately equivalent to the threshold (1:2) titer for the more subjective ACIF assay using Raji cells.

DISCUSSION

Characterization of the EBNA polypeptide and elucidation of its functions in latency and immortalization have been hampered by the low abundance of the protein in lymphoblastoid cells and a lack of specific immunological reagents: for example, EBNA is traditionally detected using EBV-seropositive human serum and complement-mediated immunofluorescence. The bacterial 28-kDa EBNA polypeptide now provides an abundant, easily purified EBNA reagent likely to be of considerable value for both clinical evaluation and for basic studies of the role of EBNA in EBV latency. The availability of this protein permitted the preparation of both rabbit polyclonal and mouse monoclonal antibodies that gave the typical EBNA staining pattern by ACIF on EBV-carrying lymphocytes and on LEK fibroblast cells expressing EBNA. The staining obtained with a pool of monoclonal antibodies was less intense than that observed with the rabbit serum and ACIF but has the advantage of being complement-independent. Both the rabbit and the individual monoclonal antibodies also specifically recognized the intact *Bam*HI fragment K-encoded EBNA polypeptide on immunoblots. The monoclonal antibodies were originally selected not for immunofluorescence but on the basis of their ability to immunoprecipitate the 28-kDa EBNA polypeptide, and they may also prove valuable as specific reagents for immunoprecipitation.

The 28-kDa EBNA polypeptide used here contains only the carboxyl-terminal one-third of the EBNA-1 protein and lacks the Gly-Gly-Ala repeats. Therefore, the potential problem of crossreactivity of the antibodies with cellular proteins containing glycine plus alanine-rich regions (15, 17) was eliminated. Furthermore, these antibodies can be used as specific probes for examining the function of the carboxyl-terminal region of the protein. Of particular relevance in this regard is the discovery that the 28-kDa, bacterially synthesized carboxyl terminus of EBNA is a sequence-specific DNA-binding domain that interacts with the plasmid maintenance region (ori_P) of the EBV genome (30).

The increased sensitivity and objectivity of an ELISA make this procedure an attractive alternative to immunofluorescence assays for determining antibody status. Previous reports of the use of ELISAs to measure anti-EBV antibody titers (27–29) noted that the most dramatic increase in sensitivity occurred for the anti-EBNA measurements. Whereas the ELISA-determined antibody levels to the EBV viral capsid antigen and early antigens were about 2- to 3-fold greater than the titers determined by immunofluorescence, anti-EBNA titers showed a 30- to 100-fold increase. In these

earlier studies, the source of EBNA antigen was either total extracts (27) or partially purified EBNA preparations (28, 29) from EBV-positive lymphoblastoid cells. Although the potential of an anti-EBNA ELISA had clearly been demonstrated, its adoption as a routine laboratory technique was restricted by the difficulty in obtaining purified EBNA in sufficient quantity for use as antigen. This limitation can be overcome by the use of the bacterially synthesized 28-kDa EBNA polypeptide since milligram quantities of this protein can be readily obtained in highly purified form.

The sensitivity of the bacterial antigen ELISA is illustrated by the finding that nasopharyngeal carcinoma patient sera with ACIF titers between 1:320 and 1:640 gave ELISA titers between 1:50,000 and 1:100,000. However, the titers of individual serum samples measured by 28-kDa EBNA ELISA were not always directly comparable to those measured by ACIF, which may be a reflection of the presence in some human sera of antibodies against other EBNAs (6, 7) and against epitopes on the amino-terminal two-thirds of *Bam*HI fragment K-encoded EBNA. A similar observation was made by Dillner *et al.* (15), who used an ELISA based on a synthetic Gly-Gly-Ala-containing polypeptide. The bacterial antigen ELISA also showed complete specificity in that EBNA titers were observed only in individuals who showed serological evidence for prior exposure to EBV. The 12 normal sera and 3 rheumatoid arthritis patient sera that were EBNA negative in this assay were also negative for the viral capsid antigen by immunofluorescence. In addition, the 28-kDa EBNA ELISA appeared to be unaffected by the presence of nonspecific anti-nuclear antibodies which prevent a determination of EBNA titer in the ACIF assay. The 28-kDa EBNA ELISA is thus both sensitive and specific. Finally, as would be necessary to validate the use of the 28-kDa EBNA polypeptide as an ELISA reagent, the overall pattern of ELISA determined anti-28-kDa EBNA titers was consistent with results obtained previously by others using ACIF (26) or the earlier ELISAs (27–29). Specifically, low EBNA titers were observed in the early stages of infectious mononucleosis, whereas nasopharyngeal carcinoma patients all had high EBNA titers and some Burkitt lymphoma patients had high EBNA titers, while others were in the normal range. The rheumatoid arthritis patients gave a similar range and distribution of anti-EBNA titers to those of seropositive but healthy individuals except for some skewing of the arthritis patient samples towards higher titers.

The anti-28-kDa EBNA ELISAs also provided some insight into the humoral immune response to EBNA. The sensitivity of the bacterial antigen ELISA measurements for anti-EBNA antibodies enabled us to quantitate anti-EBNA antibody in all individuals who were seropositive for the EBV viral capsid antigen. The anti-EBNA titers were spread over a three-orders-of-magnitude range of values, with the highest titers in the normal and rheumatoid arthritis groups being equivalent to those seen in nasopharyngeal carcinoma patient sera. We calculated that these high-titer individuals possess antibodies to EBNA at concentrations up to 1 mg/ml of serum, representing >1% of the total IgG content. We conclude from the anti-28-kDa EBNA ELISA data that EBNA, and in particular the carboxyl-terminal domain of EBNA, is extremely immunogenic in man. The high titers of anti-EBNA antibody measured by this assay raise the possibility that the degree of exposure of the immune system to EBNA, in normal individuals as well as those with ongoing disease, may have been underestimated previously because of the insensitivity of the ACIF assay for EBNA.

We are grateful to Bart Barrell and colleagues for making available the EBV *Bam*HI K-fragment nucleotide sequence prior to publication. We thank Werner Henle (The Children's Hospital, Philadelphia) for providing sera from infectious mononucleosis patients, Paul Levine (National Cancer Institute) for Burkitt lymphoma and nasopharyngeal carcinoma sera, Marc Hoffberg (The Johns Hopkins University) for rheumatoid arthritis sera, and David Levy (The Johns Hopkins School of Medicine) for control sera from normal individuals. We also thank Loretha Myers for technical assistance and Nancy Standish for preparation of the manuscript. This work was supported by Public Health Service Grants ES03131 awarded to G.M. by the National Institute of Environmental Health Sciences; CA37314 and CA30356 awarded to G.S.H. and S.D.H., respectively, by the National Cancer Institute; and GM31158 awarded to J.T.A. by the National Institute of General Medical Sciences.

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