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Biochemistry

Antibodies against a synthetic peptide identify the Epstein–Barr virus-determined nuclear antigen

(peptide synthesis/anticomplement immunofluorescence)

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ABSTRACT **Five peptides corresponding to amino acid sequences predicted from all three reading frames of the nucleotide sequence of the third internal repeat array (IR3) of the Epstein–Barr virus (EBV) genome were synthesized chemically. All five peptides elicited antipeptide antibodies in rabbits. The antiserum raised against a 14-residue copolymer of glycine and alanine gave brilliant EBV-specific nuclear staining in the anticomplement immunofluorescence (ACIF) assay, in line with the original definition of the EBV-determined nuclear antigen (EBNA) [Reedman, B. M. & Klein, G. (1973) *Int. J. Cancer* 11, 499–520]. Eight EBNA and EBV DNA-carrying lines showed nuclear staining with the antipeptide antibody, whereas five EBV DNA negative lines failed to stain. The staining pattern was more discretely punctate than the finely dispersed diffuse EBNA staining obtained with human antisera. Human EBV antibody-positive but not EBV-negative sera reacted with the synthetic peptide in an ELISA test. The peptide-specific antibodies were purified from the sera of healthy EBV-seropositive persons by affinity chromatography with the peptide. They gave an EBV-specific, brilliant punctate nuclear ACIF staining similar to that of the rabbit antipeptide antibodies. It was concluded that the glycine–alanine structure encoded by the IR3 region contains a native determinant of EBNA, detected by the ACIF test. Immunoblotting with the rabbit and human peptide-specific antibodies identified polypeptides that varied between 70 and 92 kilodaltons in size in different EBV-positive cell lines, corresponding closely to a previously identified variation pattern in the size of EBNA. In addition, rabbit antipeptide antibodies identified two cellular polypeptides, 44 and 49 kilodaltons in size.**

Epstein–Barr virus (EBV) converts normal B lymphocytes into immortalized lymphoblastoid cell lines (1, 2). Conversion is regularly accompanied by the appearance of the EBV-determined nuclear antigen (EBNA) (3). EBNA has been detected in all EBV-genome-carrying cells by anticomplement immunofluorescence (ACIF) (4), including the two EBV-carrying human tumors, Burkitt lymphoma and nasopharyngeal carcinoma (5, 6). EBNA appears at a very early state of the primary transforming interaction between the virus and the B lymphocytes already before the onset of cellular DNA replication (7). In mitotic cells EBNA remains associated with the metaphase chromosomes (4), in contrast to the papovavirus T antigens that spread to the cytoplasm. This is in line with the strong DNA-binding properties of EBNA (8).

The biochemical properties of EBNA have been studied in several laboratories. Proteins of various size classes varying from 200 to 48 kDa have been shown to react with human anti-EBNA-positive antisera (9–12). Strnad *et al.* showed that extracts of proteins of molecular size between 63 and 67 kDa were capable of inhibiting the ACIF reaction, and a correlation with an EBV-specific antigen varying in size between different EBV-carrying cell lines was suggested (12). Recent transfection studies with EBV DNA fragments have indicated that a nuclear antigen can be induced by *Bam*HI K fragment (13). The *Bam*HI K fragment contains the third internal repeat region (IR3). It consists of repeated hexa- or nonanucleotide sequences of only three nucleotide triplets: GGG, GCA, and GGA (14). Multiple homologous sequences are present on the human chromosomes, suggesting that the virus may originally have picked up this sequence from the host cell genome (15).

Different isolates of EBV contain IR3 sequences of varying length (14). The length of the IR3 sequence is directly correlated with the size of an EBV-associated nuclear antigen varying in size between 68 and 85 kDa (16).

The reading frame for transcription of this protein has recently been determined by immunizing rabbits with fusion proteins made in bacteria carrying plasmid clones containing β-galactosidase and IR3, joined in all three reading frames. The protein corresponding to reading frame 2 induced antibodies in rabbits that reacted with the IR3-related proteins on immunoblotting (17). However, EBNA reactivity of these antibodies was not proven by an ACIF reaction.

If the IR3 region codes for EBNA or one of its major components, it should be possible to induce EBNA-specific, ACIF-reactive antibodies, by immunizing rabbits with a short synthetic peptide, deduced from the DNA sequence. This approach would firmly corroborate the relationship between the products of the IR3 region and EBNA and would also confirm the recent identification of the correct reading frame. Moreover, it might provide evidence for the existence of cellular proteins containing sequences related to the IR3 region, possibly reflecting the IR3 cellular homologues (15).

Furthermore, a monospecific anti-EBNA reagent would open numerous areas for study that cannot be performed presently. These objectives were achieved in the present study.

MATERIALS AND METHODS

Cells. Cell lines were maintained as described (4). For origin of cell lines, see refs. 4 and 14. LSB-1 is derived from a healthy donor by immortalization with B95-8 virus.

ACIF. This was performed as described (4). Contrast staining was in Evans blue (0.01%) in distilled water.

Production of Peptides and Antipeptide Sera. Peptides and antipeptide antibodies were prepared as described (18).

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Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV-determined nuclear antigen; ACIF, anticomplement immunofluorescence; IR3, third internal repeat region.

Table 1. Synthetic peptides and the antibody response against them

Peptide no.	Amino acid sequence	Reading frame	Localization of corresponding sequence in the *Bam*HI K fragment, no. of amino acids in the second phase open reading frame of IR3	Rabbit antipeptide response: ELISA titer, rabbit 1/rabbit 2	Anti-EBNA response in ACIF, rabbit 1/rabbit 2
107	Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Ala-Gly-Gly-Ala-Gly-Cys*	2	150–168† 176–195 271–289	1:10,240/1:640	1:32/1:4
106	Gly-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Ala-Gly-Ala-Gly-Gly-Gly-Gly-Arg-Cys*	2	315–327	1:5,160/1:10,240	0/0
108	Cys-Arg-Ala-Arg-Gly-Arg-Gly-Arg-Gly-Arg-Gly-Glu-Lys-Arg-Pro-Met*	2	369–383	1:5,120/1:5,120	0/0
105	Cys-Lys-Ser-Gln-Gly-Glu-Arg-Ser-Trp-Thr-Trp-Arg-Lys-Glu-Ala-His*	1	369–383	1:10,240/1:10,240	0/0
109	Cys-Lys-Glu-Pro-Gly-Gly-Glu-Val-Val-Asp-Val-Glu-Lys-Arg-Gly-Pro*	3	369–383	1:40,960/1:20,480	0/0

*These residues were not in the primary amino acid sequence but were added to allow coupling to carrier.
†The sequence is repeated three times in the IR3 region of the B95-8 virus (14).

Antibody Purification. Five milligrams of peptide 107 (see Table 1) was dissolved in water and coupled to AH-Sepharose 4B (Pharmacia) as described in the manufacturer's manual.

The column was equilibrated with 150 mM NaCl/1 mM EDTA/20 mM Tris·HCl, pH 7.5 (NET buffer). Immune serum (human or rabbit) was applied and the column was washed with 30 column vol of NET buffer. Antibodies were eluted with 100 mM citrate buffer at pH 3.0. pH was adjusted to neutral with Tris base and the eluate was dialyzed against phosphate-buffered saline (P_i/NaCl).

Preparation of Antigen for Immunoblotting. Cells were thawed in NET buffer containing 5 mM mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride. Following centrifugation, the supernatant was applied to a column of double-stranded DNA-cellulose [prepared as described (19)]. The column was washed with 5 column vol of NET buffer and bound proteins were eluted with NET buffer containing 1 M NaCl.

Immunoblotting. DNA-binding proteins from four virus-carrying and two virus-negative cell lines were separated on 10% $NaDodSO_4$/polyacrylamide gels as described by Laemmli (20). Molecular weight markers were from Bio-Rad. Samples corresponding to 10^7 cells were applied to each lane. The proteins were transferred to nitrocellulose as described by Towbin *et al.* (21). The transfer was at 36 V, 1.4 A, for 1 hr at 4°C. Blots were incubated with P_i/NaCl with 0.05% Tween 20 (P_i/NaCl-T) containing 2% bovine serum albumin for 30 min at 37°C. Incubation with purified antibody (diluted 1:10 in P_i/NaCl-T with 2% bovine serum albumin) was for 60 min at 37°C. Following three 60-min washes with P_i/NaCl-T the blots were incubated for 20 min with protein A (Pharmacia) conjugated to alkaline phosphatase [prepared as described (22)]. Blots were washed as above and incubated with 20 mg of α-naphthyl phosphate and 50 mg of fast red (Sigma) in 100 ml of 100 mM Tris·HCl, pH 8.8/1 mM $MgCl_2$. The development of the color reaction was terminated after 15 min.

ELISA. This was performed as described (23). One microgram of synthetic peptide was added to each well. The conjugate was a protein A–alkaline phosphatase conjugate, prepared as described (22). The substrate was 1 mg of *o*-nitrophenyl phosphate (Sigma) per ml in a solution of 1 mM $MgCl_2$/0.1 M Tris-glycine, pH 10.4. The reaction was allowed to take place for 3 hr at 4°C. The titer was defined as the last dilution of serum to give a 405-nm absorbance of 0.2 above background level.

RESULTS

Immunogenicity of Synthetic Peptides. Each of the peptides listed in Table 1 elicited specific antibodies in rabbits as detected by the ELISA test (Table 1). The antisera were tested for their ability to react with EBNA by ACIF and immunoblotting. Only the glycine–alanine copolymer 107 elicited EBNA-specific antibodies, as described below. Fig. 1 shows that 19 of 22 EBNA-antibody-positive human sera reacted

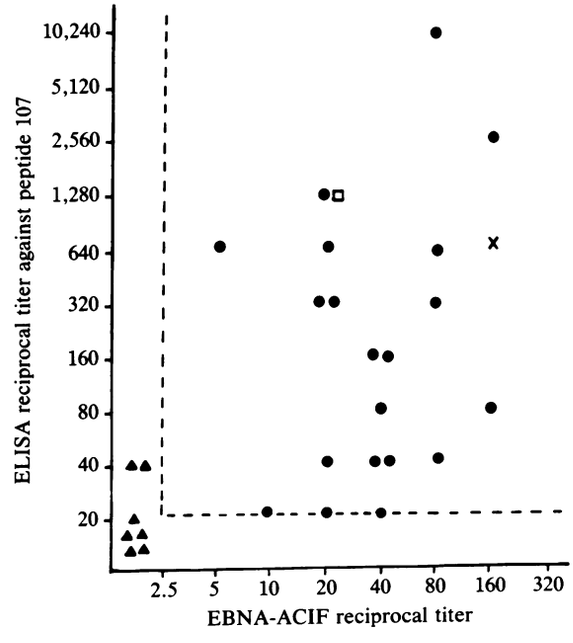

FIG. 1. EBNA-ACIF titers of human EBV-positive and EBV-negative sera against Raji cells and their titers against the synthetic peptide 107 in a direct binding ELISA test. ▲, EBV-negative sera; ●, EBV-positive sera; □ and ×, two EBV-positive sera that were used for affinity purification.

with this peptide in the ELISA test, whereas 7 EBV-negative sera did not react or gave very weak reactions.

Purification of Peptide-Specific Antibodies from Human Sera. We selected two sera with antibody titers of 1:1280 and 1:640. The anti-EBNA titers in ACIF were 1:20 and 1:160, respectively (Fig. 1). Specific antibodies were isolated on an affinity column of AH-Sepharose coupled to the peptide. The recovery was >90%, as measured in the ELISA test. The anti-EBNA titers in ACIF were reduced to 1:10 for both sera.

ACIF. Preimmune and immune sera from 10 rabbits immunized with the five synthetic peptides listed in Table 1 were tested for their ability to stain the nuclei of EBV-carrying, compared to EBV-negative, human lymphoblastoid cell lines. The sera derived from the rabbits immunized with peptide 107 showed nuclear staining. Following affinity purification with peptide 107, the staining reaction was specific for the EBV-carrying lines (Table 2; Fig. 2). The staining pattern was unusual. Instead of the characteristic brilliant, diffuse nuclear staining, a stippled pattern was obtained with large brilliant dots and spots (Fig. 2 *B* and *C*). The staining was prevented by preincubation with the synthetic peptide (Fig. 2*D*). There was a certain variation in staining intensity of the different cell lines with the antipeptide antisera. For instance, Raji and Namalwa showed a relatively weak nuclear staining, whereas P3HR-1 and Cherry gave a brilliant staining (Fig. 2 *B* and *C*; Table 2). The human antibodies that were affinity purified with peptide 107 gave a stippled staining pattern similar to that of the rabbit anti-107 antibodies (Fig. 2*E*). This staining was also inhibited by preincubation with the synthetic peptide (Fig. 2*F*). The human antibodies that failed to bind to the affinity column gave a very different, brilliant and diffuse, finely granular nuclear fluorescence (Fig. 2*H*) that resembled the usual EBNA pattern obtained with human sera (Fig. 2*A*). The EBNA staining of human sera was not visibly affected by preincubation with synthetic peptide 107.

Immunoblotting. Blots incubated with either rabbit or human anti-EBNA antibodies purified by affinity chromatography with peptide 107 gave specific bands that varied in molecular weight between different virus-carrying cell lines in a similar manner as described by Strnad *et al.* (12). These bands were only detected in EBV-carrying, but not EBV-negative, cell lines and could be blocked by the peptide.

Raji cells contained the smallest (70 kDa) and Cherry cells contained the largest polypeptide (92 kDa). P3HR-1 and B95-8 contained EBV-specific 76-kDa and 78-kDa polypeptides, respectively.

Table 2. Nuclear ACIF reaction with affinity-purified rabbit and human sera

Cell	EBV carrier state	ACIF with: EBNA-positive human sera diluted 1:4	ACIF with: Affinity-purified rabbit antibodies to peptide 107 diluted 1:4	ACIF with: Affinity-purified human antibodies diluted 1:4
BL 30	Negative	−	−	−
BJAB	Negative	−	−	−
Ramos	Negative	−	−	−
Loukes	Negative	−	−	−
BL 2	Negative	−	−	−
Raji	Positive	+++	++	++
LSB-1	Positive	+++	+	+
P3HR-1	Positive	+++	+++	+++
Namalwa	Positive	+++	+	+
Daudi	Positive	+++	+++	+++
B95-8	Positive	+++	+++	+++
Cherry	Positive	+++	+++	+++
Jijoye	Positive	+++	+++	+++

Both the human and rabbit affinity-purified antisera recognized some non-EBV-specific bands as well. Bands in the 34- to 31-kDa size category were detected in both EBV-carrying and EBV-negative cell lines (Fig. 3 *A–C*). They could not be blocked by preincubation with the peptide (Fig. 3*B*) but could be removed by preabsorption with EBV-negative Ramos cells (Fig. 3*D*).

The rabbit but not the human affinity-purified antibodies detected two prominent polypeptides of 44 and 49 kDa (Fig. 3*A*). They did not react with the preimmune rabbit sera (not shown). Preincubation with peptide 107 abolished the reaction against these polypeptides (Fig. 3*B*). Preabsorption of the rabbit antipeptide sera with EBV-negative Ramos cells absorbed the reactivity against the 44- and 49-kDa proteins, without notably diminishing the reactivity against the EBV-specific polypeptides (not shown).

DISCUSSION

Our results show that antipeptide antibodies raised against a 14-residue synthetic glycine–alanine copolymer deduced from the nucleotide sequence of the third internal repeat array (IR3) of the *Bam*HI K fragment of the EBV genome are reactive with a native determinant of EBNA, also detected in the ACIF assay. These data agree with the recent findings by Hennessy and Kieff (17), showing that a glycine–alanine copolymer encoded by IR3 in the second reading frame is a part of an EBV-determined nuclear antigen.

Peptide-specific antibodies often react with the native protein molecule, even in cases in which the sequence does not correspond to an immunogen of the folded protein (24). The opposite is not necessarily true, in that antibodies directed against the native protein molecule usually fail to react with derived synthetic peptides. Our finding that EBNA-antibody-positive human sera react with the glycine–alanine copolymer in an ELISA test, whereas antibody-negative sera fail to react, shows that the synthetic peptide represents a determinant of the native EBNA protein. The regular occurrence of antibodies against this determinant, present in 19 of 22 sera from EBV-infected individuals, is unusual for synthetic peptides and shows that the copolymer is a regularly immunogenic determinant of the native protein.

Human and rabbit peptide 107 affinity-purified sera recognize an EBV-specific antigen varying in size between 70 and 92 kDa on immunoblots. The size variation is similar to recent reports on EBV-specific polypeptides detected by immunoblotting in EBV-carrying, virus nonproducer lines (12, 16, 17, 25). Our findings confirm these studies and extend them in showing that the proteins detected by immunoblotting can give an EBNA-specific ACIF reaction.

The rabbit, but not the human, sera also detected two DNA-binding polypeptides of 44 and 49 kDa in size. The reaction was blocked by the peptide. It can be surmised that the synthetic peptide shares an antigenic determinant with these cellular proteins. Although such crossreactivity could also occur by chance, a true crossreactivity is more likely, due to the fact that the cellular genome contains multiple sequences homologous to IR3. The absence of antibodies against these polypeptides in human sera would be in line with the possibility that these are normal cellular proteins. The EBV specificity of the polypeptides in the 70- to 92-kDa size class can be reconciled with the existence of homologues transcribed in the same reading frame if the cellular IR3 gene products differ from the viral IR3 gene products in their tertiary structure. Both the human and rabbit affinity-purified sera also reacted with polypeptides in the molecular size range between 31 and 34 kDa. This reaction was not blocked by the peptide but could be absorbed with EBV-

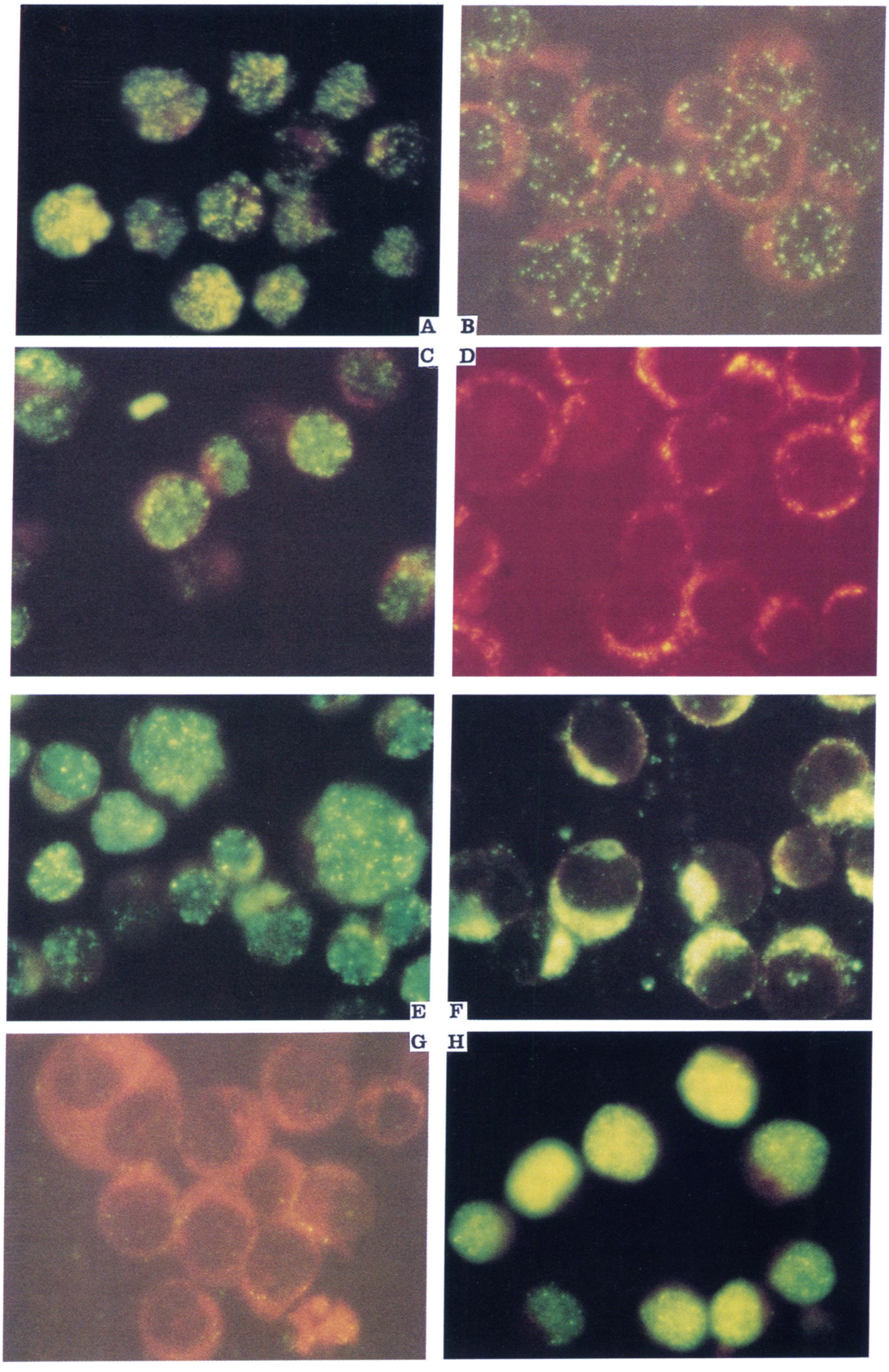

FIG. 2. ACIF with human EBNA-positive sera, Raji cells (*A*); rabbit affinity-purified antipeptide antibodies, Raji cells (*B*); rabbit affinity-purified antipeptide antibodies, P3HR-1 cells (*C*); rabbit affinity-purified antipeptide antibodies after incubation with the synthetic peptide, P3HR-1 cells (*D*); affinity-purified human serum, P3HR-1 cells (*E*); affinity-purified human serum after preincubation with the synthetic peptide, P3HR-1 cells (*F*); affinity-purified rabbit anti-peptide antibodies, Ramos cells (*G*); and human serum that failed to bind to the peptide affinity column, P3HR-1 cells (*H*). (*A–H*, ×400.)

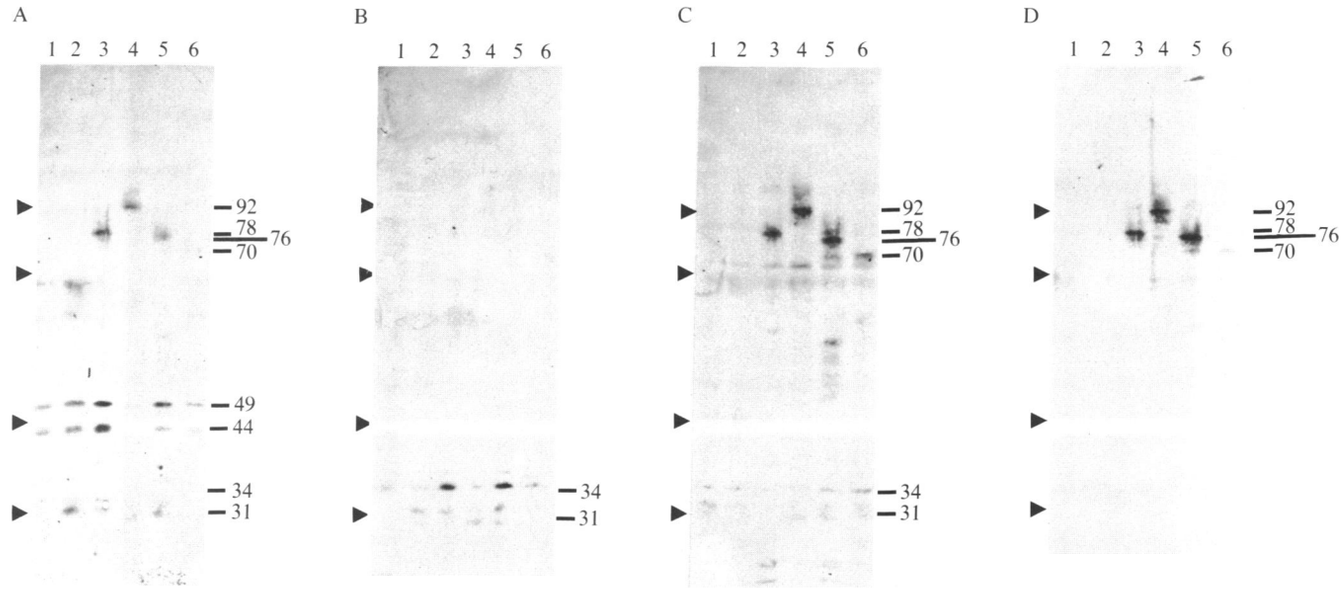

FIG. 3. Immunoblotting with rabbit affinity-purified antipeptide antibodies (*A*), rabbit affinity-purified antipeptide antibodies after preincubation with the synthetic peptide (*B*), affinity-purified human serum (*C*), and affinity-purified human serum after preabsorption with EBV-negative Ramos cells (*D*). Molecular size is given in kDa. Arrowheads indicate positions of the molecular weight markers. EBV-negative cell lines: lane 1, BJAB, and lane 2, Loukes. EBV-carrying cell lines: lane 3, B95-8; lane 4, Cherry; lane 5, P3HR-1; and lane 6, Raji.

negative cells. The antibodies against these proteins may have bound to the affinity column nonspecifically, as has been seen with rabbit antipeptide sera in other systems (26).

Of the two types of antisera against EBNA presented in this study, the human affinity-purified antibodies are likely to be the most useful. They have the advantage that large amounts of antibodies are easily purified and that the antibodies only gave peptide-specific reaction against EBNA. However, both types of antisera can be made monospecific for EBNA by preincubation with extracts of EBV-negative cells. The availability of monospecific antisera against EBNA will be useful for further characterization of EBNA.

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