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EPSTEIN-BARR VIRUS (EBV) ANTIGENIC DETERMINANTS ON SUBUNITS OF THE EBV DETERMINED NUCLEAR ANTIGEN (EBNA)

Danièle HENTZEN[1], Gilbert M. LENOIR[2], Marie-Claude BERTHELON[2] and Jacques DAILLIE[1]

1 Département de Biologie générale et appliquée, Université Claude-Bernard Lyon I, 69622 Villeurbanne cédex, France

2 International Agency for Research on Cancer, 69372 Lyon cédex 2, France

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SUMMARY: In order to characterize the substructure of the Epstein-Barr virus determined nuclear antigen (EBNA) which is considered to have a molecular weight of 180 K in its native form, we have examined the antigenic specificity of the polypeptides obtained after denaturation of this molecule. Two procedures were employed; treatment by sodium dodecyl sulfate (SDS) and heat followed by gel electrophoresis, or denaturation by guanidine hydrochloride followed by gel filtration, which allowed us to detect a specific antigenic activity in the 50 K region, following dialysis. The denatured molecules could be reassociated into larger molecules (50 to 180 K) which retain the property of binding to fixed nuclei, as does native EBNA. These results indicate that EBNA has a polymeric structure and that 50 K subunits carry the antigenic determinants.

INTRODUCTION

The Epstein-Barr virus (EBV) determined nuclear antigen (EBNA) is present in all EBV genome carrying lymphoid cells whether or not they produce viral particles (1). This antigen can also be regularly detected in human tumour cells obtained from patients either with Burkitt's lymphoma (BL) or with nasopharyngeal carcinoma (NPC) (2, 3). In common with other nuclear antigens induced by oncogenic DNA viruses such as the T-antigens of adenoviruses, polyoma virus and simian virus 40, EBNA is thought to have a role in establishing and/or maintaining the cellular transformed state, and in controlling viral genome expression. The structure of this nuclear protein with an apparent molecular weight

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(M.W.) of approximately 180 K* in its native form (4, 5, 6), is at present not clearly defined. The results of dissociation studies indicate that EBNA is made up of polypeptides of M.W. ranging from 50 to 100 K, on which immunological reactivity could no longer be detected (7, 8).

In order to further elucidate the subunit structure of EBNA, we have attempted to denature EBNA with detergents or guanidine hydrochloride and then to separate the dissociated molecules according to size and to determine their antigenic activity by complement fixation (CF) or anti-complement immunofluorescence (ACIF).

MATERIAL AND METHODS

Cells: EBNA was extracted from the non producer line Raji derived from an EBV positive African BL. In this line, 100% of the cells express EBNA while early antigens (EA) are detected in less than 0.1 % of the cells cultivated.

Cell lines were grown in suspension cultures at 37°C in RPMI 1640 medium (Grand Island Biological Co) supplemented with 10% foetal calf serum, penicillin (100 units/ml) and streptomycin (100 µg/ml).

Sera: The anti-EBNA positive sera used in this study were obtained from normal EBV-infected individuals and have no anti-EA activity. They have anti-EBNA titres of 1:640 and CF titres of 1:80 when tested on Raji smears or with Raji extracts respectively. They do not have any anti-complement activity. Anti-EBV negative sera were used as controls.

CF and ACIF tests: The complement fixation test (CF) was carried out according to the microtiter technique of Sever (9) using 2 volumes of guinea pig complement per well. Each dilution of antigen was tested for anti-complement activity.

The anti-complement immunofluorescence test (ACIF) was performed as described by Reedman and Klein (1).

Acid-fixed nuclear binding assay: The EBNA activity was also tested by the acid-fixed nuclear binding (AFNB) assay as described by Ohno *et al.* (6) and modified by Hirsch (10). Briefly, EBV genome negative BJAB cells were fixed with methanol-acetic acid, treated with 0.6 M NaCl and washed with distilled water. 100 µl of the antigen solution (.6 mg/ml) was added per fixed smear preparation, incubated for 1 hour at 20°C, washed with four changes of adsorption buffer (10 mM Phosphate buffer pH 6.0 containing 50 mM NaCl, 1 mM-β-mercaptoethanol and 5% glycerol) at room temperature for 1 hour. The samples were then washed with distilled water and tested for EBNA by the ACIF technique.

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* K used for 1000 daltons

Preparation of EBNA extracts: 2.10^9 frozen cells were thawed and suspended in a final volume of 25 ml of 20 mM Tris HCl buffer pH 7, DTT 1 mM, EDTA 1 mM, glycerol 10%, NaCl 350 mM, sonicated 4 times at 50 W for 30 sec, slowly agitated in ice for 30 min. and centrifuged at 10,000 RPM. An aliquot of the supernatant was dialysed against 10 mM Tris HCl buffer pH 7, to test the CF activity. For the guanidine hydrochloride denaturation experiments, the EBNA extract was partly purified before treatment, by gel filtration on a sepharose 6B column in 10 mM Tris HCl buffer pH 7; the CF positive fractions were pooled dialysed against 1 mM Tris HCl buffer pH 7 and concentrated by lyophilisation.

SDS polyacrylamide gel electrophoresis: Gels containing 6 % (stacking gel) and 11 % acrylamide were prepared according to Laemmli (11).

For analytical purpose, we used slabs of 0.75 x 120 x 140 mm with twenty 4 mm-wide slots. For preparative purpose, we used slabs of 3 x 120 x 140 mm with one large slot of 100 mm wide and 2 small ones of 4 mm, one to be filled with M.W. markers, the other with an aliquot of the EBNA extract as an analytical control of the electrophoresis.

Proteins were denatured in 0.0625 M Tris HCl buffer pH 6.8 containing 2 % SDS, 5 % 2-mercaptoethanol, 10 % glycerol and 0.001 % bromophenol blue as the dye. The samples were then heated by immersion in boiling water for 2 min. The final volume was 20 µl for analytical samples and 2.5 ml for preparative ones. After migration, analytical gels were fixed and colored overnight with a solution of 25% isopropanol, 10 % acetic acid and 0.025 % coomassie blue. They were destained in a solution of 10 % isopropanol and 10 % acetic acid, followed by an overnight treatment with a solution of 1 % glycerol, 25 % methanol which facilitates the drying of the gel.

The preparative gel was treated as follows: the regions of the gel used for M.W. estimation were cut and treated like the analytical gels. The other part was sliced into eight cuts of gel parallel to the front line. The proteins of each fragment were extracted from the gel by electrophoresis in an ISCO Model 1750 sample concentrator, as previously described (12). Each fraction was carefully dialysed against 1 mM Tris HCl buffer pH 7 prior to carrying out the CF test.

8 M Guanidine hydrochloride treatment and chromatography on sepharose CL-6 B: The prepurified and lyophilised EBNA preparation was dissolved in 10 mM Tris HCl buffer pH 7 denatured by addition of guanidine hydrochloride(8 M) and chromatographed on a sepharose CL-6 B column in presence of 2 mM Tris HCl buffer pH 7 containing 6 M guanidine hydrochloride. The resulting fractions were desalted on sephadex G 25, dialysed against 1 mM Tris HCl buffer pH 7 and submitted to CF and ACIF tests. Following this treatment, around 50% of the antigenic activity were recovered.

The fractions which gave a positive CF response were pooled, dialysed against distilled water, concentrated by lyophilisation, dissolved in 10 mM Tris HCl buffer pH 7 containing 100 mM NaCl and rechromatographed on a sepharose CL-6 B column in this buffer.

The molecular weight markers were cochromatographed with the EBNA extract and detected either by radioactivity in the case of I^{125} labelled bovine serum albumin (BSA M.W. = 68 K) and ovalbumin (M.W. = 45 K), or by its enzymatic activity for E. Coli alkaline phosphatase (M.W. = 89 K).

RESULTS

Antigenic activity of dissociated EBNA following SDS-polyacrylamide gel electrophoresis

After extraction of the polypeptides from the different gel slices and dialysis, one aliquot of each fraction was submitted to the CF test while another aliquot was analysed by SDS-polyacrylamide gel electrophoresis. Figure 1 shows an example of a protein fractionation using this technique.

Consistent antigenic activity was detected by CF in one fraction (track g). The molecular weight of the polypeptides migrating in this zone corresponded to approximately 50 K. In some experiments, however, CF activity was recovered in the zone migrating just below ovalbumin (Figure 1, track i). In all cases, CF activity was low and detected

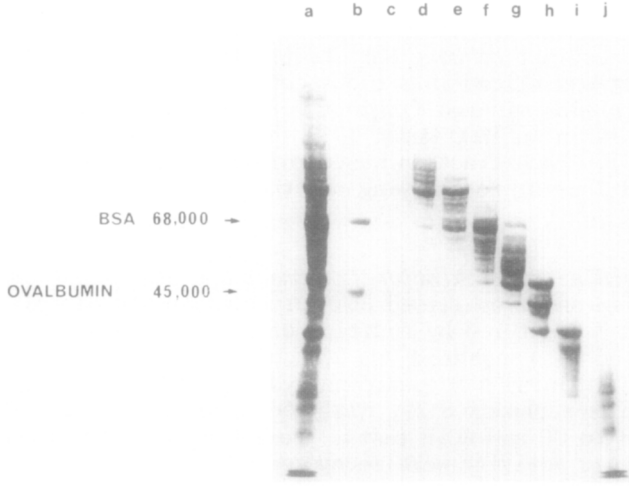


Figure 1 : SDS-polyacrylamide slab gel electrophoresis of protein fractions obtained from a crude EBNA extract and separated according to their M.W. by a preparative electrophoresis. The samples were applied to a 11% polyacrylamide gel in 0.1% SDS with a 6% stacking gel. Track (a) contains crude EBNA extract from Raji cells used for the preparative electrophoresis. Two marker proteins were run in track (b): bovine serum albumin (68,000) and ovalbumin (45,000). The eight following tracks (c-j) contain one of the eight fractions extracted from the preparative gel.

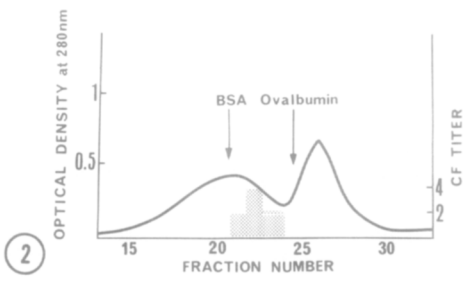
only in undiluted samples. Negative results were obtained with the less sensitive acid fixed nuclear binding (AFNB) technique. A preparative gel was overloaded with the aim of increasing the CF response, but this only resulted in increasing the level of contamination of each fraction by proteins of adjacent areas.

We therefore looked for a technique which would allow a better recovery of antigenic activity.

Analysis of EBNA subunits by chromatography on sepharose CL-6 B in the presence of 6 M guanidine hydrochloride

The peak corresponding to 180 K native EBNA molecules obtained after a preparative sepharose 6 B gel filtration (4) was dissociated by 8 M guanidine hydrochloride and rechromatographed on sepharose 6 B in the presence of 6 M guanidine hydrochloride. After dialysis and concentration, CF activity could be recovered from fractions which eluted in a peak situated between the two protein markers BSA (68 K) and ovalbumin (45 K) (Figure 2). In agreement with the data obtained by SDS denaturation, this type of denaturation and fractionation also suggested the presence of approximately 50 K subunits.

The fractions obtained after guanidine hydrochloride denaturation contained sufficient CF activity to convert *in vitro* EBNA negative BJAB fixed nuclei into positive nuclei as detected by ACIF (6, 10). Both CF and ACIF assays were performed following dialysis against 10 mM Tris HCl buffer in order to eliminate the guanidine hydrochloride. This treatment could allow renaturation of 50 K subunits into larger molecules. In order to evaluate the M.W. of renatured molecules, the dialysed antigenically active fractions were rechromatographed on sepharose 6 B under non dissociating conditions. CF activity was then detected in fractions corresponding to M.W. ranging from 50 K to 180 K (Figure 3).



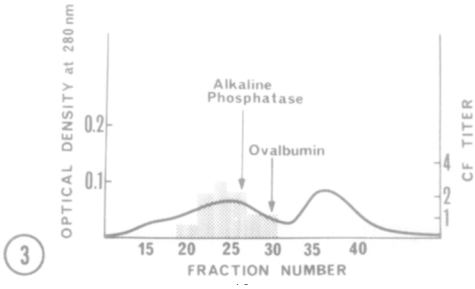


Figure 2 : Chromatography on sepharose CL-6 B in presence of 6 M guanidine hydrochloride of a partially purified EBNA fraction. The sample was treated with 8 M guanidine for 1 h and applied to the column (1.6 x 90 cm). Fractions of 4 ml eluted from the column with 6 M guanidine in 2.10^{-3} M Tris HCL uffer pH 7 were concentrated, dialysed and tested for CF expressed as reciprocal titres of antigen dilution. Optical density was monitored at 280 nm. The molecular weight markers were chromatographed with the EBNA extract and detected either by radioactivity in the case of I^{125} labelled bovine serum albumin (BSA,M.W. = 68,000) and ovalbumin (M.W. = 45,000).

Figure 3 : Chromatography of fractions (21-24)illustrated by Figure 2 on a sepharose 6 B column (1.5 x 30 cm) in 10 mM Tris HCl buffer pH 7. 1 ml fractions were concentrated and tested for CF activity. The molecular weight markers were ovalbumin (M.W. = 45,000) and E. Coli alkaline phosphatase (M.W. = 89,000). Symbols as in Figure 2.

DISCUSSION

In this study, in order to obtain information on the substructural organisation of EBNA, we have estimated the antigenic activity of denatured molecules both by CF and ACIF, in addition to determining the M.W. of the subunits obtained.

After heat denaturation in the presence of SDS and mercaptoethanol followed by polyacrylamide gel electrophoresis under denaturing conditions, in general a single fraction located in the 50 K M.W. region, was found to give a positive CF response. Similar results were obtained when denaturation was achieved by 8 M guanidine hydrochloride followed by fractionation by gel filtration in presence of 6 M guanidine hydrochloride. In addition to confirming previous results, the technique we used has enabled us to obtain sufficient amount of material to allow detection of antigenic activity by both CF and ACIF assays.

In some cases, following the SDS denaturation procedure, antigenic activity was detected in a fraction located below ovalbumin. It is premature to conclude whether this smaller polypeptide, although exhibiting some antigenic activity, corresponds to a significant subunit or to a degradation product.

Rechromatography on sepharose 6 B of dialysed 50 K fractions showed that a reassociated form of EBNA can be obtained from such fractions. However, renaturation of the 50 K subunits in a 180 K native molecule remains partial since the antigenic activity is located from the 180 K down to the 50 K region. Nevertheless, our results indicate that the antigenic activity can be recovered after denaturation and fractionation of the subunits. This suggests that the molecule is rather stable, as is also indicated by its high heat resistance, as reported by others (5, 13). However, possible conformational changes of the protein occurring during purification, denaturation or renaturation could explain the low antigenic recovery after such experiments.

Other authors have reported that EBNA can be dissociated into subunits having a M.W. of 70 K or 100 K (5, 14). However, we were unable to detect CF positive fractions in this M.W. range.

We can conclude that the major immunological reactivity of EBNA is related to 50 K subunits. It is not yet possible to say whether the antigenic activity requires reassociation of the subunits, because dialysis is required before the CF or ACIF tests can be performed.

These results are in agreement with the data obtained by Luka *et al.* (7) and Baron and Strominger (13) who reported that, following purification, EBNA was constituted mainly by polypeptides of M.W. ranging from 48 K

to 53 K, but they were not able to detect antigenic activity on these purified subunits. It is of interest to note that, recently, Luka and Klein reported (Fourth Cold Spring Harbor Meeting on Herpesviruses 1979, Abstracts, p. 197) that EBNA may be constituted of two distinct polypeptides of 48 K and 53 K respectively. They also reported that the antigenic activity may be carried by only the 48 K subunit, and not by the 53 K polypeptide, the latter being present in extracts of the EBV negative Burkitt lymphoma lines Ramos and BJAB. Our data do not exclude the existence of different types of subunits around 50 K, with only some of them carrying the relevant antigenic determinant.

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