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Biochemistry

Antibodies to the Epstein–Barr virus nuclear antigen and to rheumatoid arthritis nuclear antigen identify the same polypeptide

(protein blots/autoimmunity/B lymphocytes/DNA-binding proteins/complement fixation)

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ABSTRACT **Patients with seropositive rheumatoid arthritis (RA) have elevated titers of precipitating antibody toward an antigen designated RA nuclear antigen (RANA). Anti-RANA reactivity has been associated with prior Epstein–Barr virus (EBV) infection. Using the protein blot technique, we have identified, in extracts of WI-L2, an EBV$^+$ nonproducer B-lymphoblast line, a M_r 80,000 polypeptide that is reactive with anti-RANA-containing sera. This same polypeptide can be recovered from RANA precipitin bands. The M_r 80,000 polypeptide appears to be EBV-associated, as a homologous antigen was detected in two other EBV$^+$ cell lines, Daudi and Raji, but was not present in three EBV$^-$ human cell lines tested, HeLa, BJAB, and Ramos. Anti-RANA antibodies and antibodies reactive with the M_r 80,000 polypeptide also appear coincidently in the sera of individuals exhibiting an EBV infection (infectious mononucleosis). Further analysis of the RANA-associated M_r 80,000 polypeptide suggested its identity with the previously recognized EBV-associated nuclear antigen designated EBNA. The M_r 80,000 antigen shares with EBNA the properties of being a heat-stable, DNA binding protein. EBNA is traditionally assayed by a complement fixation reaction and anti-M_r 80,000 antibodies were shown to be reactive when a complement fixation assay was used in the immunoblot. Finally, when the M_r 80,000 antigen was used to absorb an anti-RANA/anti-EBNA serum, both antibodies were reduced.**

The rheumatoid arthritis nuclear antigen (RANA) was first identified in WI-L2 cells by a precipitating antibody seen in the sera of most rheumatoid arthritis (RA) patients but uncommonly in normal sera (1, 2). RANA was shown to be a nuclear antigen expressed exclusively in Epstein–Barr virus (EBV) genome-positive B-lymphoblast lines (3). With more sensitive assays, anti-RANA can now be detected in many normal sera, although in lower titer than in RA sera (4–7), and also during recovery from acute infectious mononucleosis (IM) (5).

The relation between RANA and the Epstein–Barr nuclear antigen (EBNA) (8) has remained a matter of some uncertainty. RANA was originally detected in extracts of EBV-infected cells by precipitation reactions but has also been detected in an assay using heat fixation and anti-IgG immunofluorescence (IF) (1, 2). EBNA was originally assayed on acetone-fixed cells by anticomplement immunofluorescence (ACIF) and later by complement fixation with soluble cell extracts (8, 9). EBNA staining typically displays a plaque-like or diffuse stain confined to the nucleus (8), whereas RANA is more granular or speckled and sometimes extends into the cytoplasm (2). RANA and EBNA staining patterns are maximally expressed at different times in the cell cycle of WI-L2 cells and they are differentially expressed in somatic cell hybrids (10, 11). Significant differences in anti-EBNA and anti-RANA titers have been reported for individual sera—notably, those from Burkitt lymphoma patients (3). However, antibodies reactive with EBNA and RANA have been shown to arise roughly in parallel after EBV infection (5), and anti-EBNA and anti-RANA titers in individual RA patients and normal adults are closely correlated (4, 6). Only EBNA has been partially purified and characterized; however, the EBNA sizes reported range from M_r 48,000 to M_r 100,000 (12–15). In the present study we have identified a polymorphic polypeptide that varies in its M_r about 80,000 and is reactive with both anti-RANA and anti-EBNA antibodies.

MATERIALS AND METHODS

Cell Lines. The majority of experiments described employed WI-L2 cells, an EBV genome-positive nonproducer B-lymphoblast line, derived from a patient with hereditary spherocytic anemia (16). Raji and Daudi are nonproducer EBV$^+$ B-cell lines from Burkitt lymphoma patients. BJAB and Ramos are B-lymphocyte cultures derived from patients with African and American Burkitt lymphomas but do not carry EBV DNA. Cultures were grown in RPMI 1640 supplemented with 2 mM L-glutamine and 10% fetal calf serum. Suspension cultures of HeLa cells, grown in minimal essential medium with 5% calf or fetal calf serum, were kindly provided by R. Seale and J. Kates.

Nuclear Isolation and Extraction. After washing in phosphate-buffered saline (150 mM NaCl/10 mM sodium phosphate, pH 7.4) containing 0.2 mM phenylmethylsulfonyl fluoride, cells were swollen for 5 min in reticulocyte standard buffer (RSB; 10 mM NaCl/10 mM Tris·HCl, pH 7.4/1.5 mM $MgCl_2$/0.2 mM phenylmethylsulfonyl fluoride). The cells were pelleted and homogenized in RSB containing 0.2% Nonidet P-40; then nuclei were sonicated in 3–5 vol of the same buffer adjusted to 0.2–0.35 M NaCl. After 30 min on ice, the nuclear extract was centrifuged at 10,000 × *g* and used as a source of antigen. When heat fractionation was used, the extract was diluted to *ca.* 0.15 M NaCl and rapidly heated to 75°C, held for 10 min, chilled, and centrifuged at 20,000 × *g* for 15 min.

Immunoblotting Procedures. Nuclear extracts were concentrated by overnight precipitation with 2 vol of ethanol at −20°C and then were dissolved in sample buffer for $NaDodSO_4$ gels. Gels were cast and run according to the procedure of Laemmli (17), applying 100 μg of protein per cm of gel. After electro-

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Abbreviations: ACIF, anticomplement immunofluorescence; EBNA, Epstein–Barr nuclear antigen; EBV, Epstein–Barr virus; IF, anti-IgG immunofluorescence; RA, rheumatoid arthritis; RANA, RA nuclear antigen; RNP, ribonucleoprotein; SLE, systemic lupus erythematosus; VCA, viral capsid antigen; IM, infectious mononucleosis.

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phoresis, gels were transferred electrophoretically to nitrocellulose sheets by the procedure of Towbin *et al.* (18) with a Bio-Rad Trans-Blot apparatus at 70 V for 2–3 hr in 12.5 mM Tris hydroxide/96 mM glycine buffer with 20% methanol. Transferred molecular weight marker proteins were stained with amido black. Lanes to be reacted with sera were first saturated for 1 hr in 5% bovine serum albumin/5% ovalbumin/P_i/NaCl and then were cut into vertical strips; each was reacted with a different serum (diluted a minimum of 1:20 in 2% bovine serum albumin/2% ovalbumin/P_i/NaCl) for 1 hr at room temperature. For routine indirect assay, the strips were incubated for 1 hr in ^{125}I-labeled rabbit anti-human IgG (γ specific) diluted to 2.5×10^5 cpm/ml. For complement fixation, the reaction was undertaken at 37°C with sera diluted in barbital-buffered saline/bovine serum albumin/ovalbumin with 2.5 mM Mg^{2+}- and 0.75 mM Ca^{2+}-containing dilute viral capsid antigen-negative (VCA^-) serum as a source of complement. Bound complement was then scored by reaction with iodinated anti-C4. After a final wash, the strips were dried and exposed to x-ray film with an intensifying screen for 12–48 hr at −70°C.

Sera. All sera positive for anti-RANA and anti-EBNA were obtained from a serum bank maintained in the Scripps Clinical Immunology Laboratory. Differentiation of anti-RANA immunoprecipitates from other anticell or antinuclear antibodies was made in all sera by showing lines of identity of the anti-RANA precipitate with that of a prototype serum (2). VCA^- sera were also obtained from the serum bank; these sera were negative for anti-EBNA by ACIF and for anti-RANA by IF. Samples from IM patients were provided from an earlier study (5).

RESULTS

Anti-EBNA/Anti-RANA Sera Identify an Immunoreactive Polypeptide in EBV-Infected Cells. Nuclear and total cellular extracts from WI-L2 cells, an EBV-genome positive nonproducer line with which RANA was first identified, were screened by the immunoblot technique against sera containing anti-RANA activity to identify individual polypeptides that might correspond to RANA. A single trypsin-sensitive polypeptide of M_r ≈80,000 was readily detected. The few human sera nonreactive for the M_r 80,000 antigen were from individuals negative for antibodies to EBV.

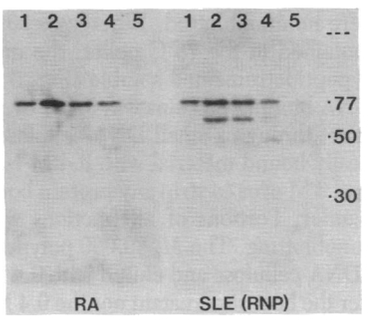

FIG. 1. Distribution of the M_r 80,000 antigen in cellular extracts of WI-L2 cells. Nuclei were isolated from WI-L2 cells and disrupted by sonication in 0.2 M NaCl. After removal of the low-salt solubilized nucleoplasmic fraction, the residual nuclear debris was sequentially extracted with 0.35 M and 0.5 M NaCl (later pooled with high-salt/DNase fraction) and digested with DNase I in 0.5 M NaCl. The remaining material was then solubilized in $NaDodSO_4$. Aliquots of each extract, including the crude cytoplasmic fraction, were precipitated with ethanol and the proteins were resolved in parallel sets by electrophoresis through 7.5% acrylamide/$NaDodSO_4$ gels. Nitrocellulose replicas were probed with anti-RANA-RA serum (*Left*) and anti-RNP/anti-RANA serum (*Right*) from a SLE patient. Lanes 1, cytoplasmic fraction; lanes 2, 0.2 M NaCl nuclear extract; lanes 3, 0.35 M NaCl extract; lanes 4, 0.5 M NaCl/DNase supernatant; and lanes 5, final $NaDodSO_4$ extraction. Molecular weights are shown as $M_r \times 10^{-3}$.

The anti-RANA sera available all had anti-EBNA reactivity in roughly equivalent titer, and we tested >20 of these sera obtained from normal persons and RA or systemic lupus erythematosus (SLE) patients. However, only the M_r 80,000 polypeptide was identified in common by all sera. Because many proteins—e.g., nucleolar or chromosomal proteins—are not readily solubilized by sonication, an examination was made of our extraction conditions to search for the missing second antigen. Cells were separated into cytoplasmic and nuclear fractions; the nuclei were sonicated in 0.2 M NaCl, then extracted with 0.35 M and 0.5 M salt, and digested with DNase in high salt, and the final nuclear debris was dissociated with $NaDodSO_4$. By subsequent immunoblot assay (Fig. 1 *Left*) only a single M_r 80,000 polypeptide could be detected in any fraction (with the exception of minor inconsistently sized proteolytic fragments in the DNase incubated sample). As much as 60% of the total M_r 80,000 antigen was found in the cytoplasmic fraction. It is uncertain whether this represents leakage during nuclear isolation; however, a parallel blot reacted with anti-ribonucleoprotein (anti-RNP) serum from an anti-$RANA^+$ SLE patient exhibited little gross leakage of the M_r 70,000 (U1) RNP autoantigen (19) (Fig. 1 *Right*). Routinely we employ direct sonication of cells or nuclei in buffered 0.35 M NaCl.

The available evidence indicates that the expression of RANA, like EBNA, is restricted to EBV-infected B lymphocytes. Therefore, we examined cell lines lacking the EBV genome, including HeLa, BJAB, and Ramos, and two additional EBV^+ lines, Daudi and Raji. Each extract was reacted with three different sera, including two prototype anti-RANA sera (VCA^+, $EBNA^+$, $RANA^+$) from RA patients and a VCA^+ serum from a SLE patient exhibiting antibodies against the ubiquitous M_r 70,000 (U1) RNP antigen (19) to serve as an internal control for the extraction and immunoblotting procedures. Each EBV^+ line, but not the EBV^- lines, expressed but a single major polypeptide identified in common by all VCA^+ sera; however, the homologous antigen varied from M_r 80,000 in WI-L2 to M_r 79,000 in Daudi and M_r 72,000 in Raji (Fig. 2). Additional faint bands were occasionally seen in the various EBV^+ extracts but were not recognized in common by all sera. The experiments described in this report focus on the M_r 80,000 antigen in WI-L2

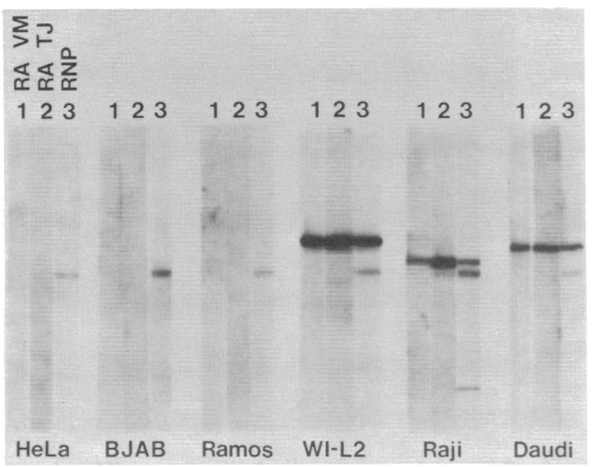

FIG. 2. Assay for immunoreactive protein in various EBV^+ and EBV^- cell lines. Nuclear extracts were prepared from three EBV^- lines, HeLa, BJAB, and Ramos, and from three EBV^+ lines, WI-L2, Raji, and Daudi. The nuclear proteins were immunoblotted and probed with two different RA sera (VM and TJ) (lanes 1 and 2) and an anti-(U1) RNP SLE serum also exhibiting anti-RANA, anti-EBNA reactivity (lanes 3). Detection was with iodinated anti-IgG.

cells; a detailed description of the molecular weight variation among various B-cell lines will be presented elsewhere.

Antibodies Reactive with the M_r 80,000 Polypeptide in WI-L2 Cells Arise After EBV Infections. The association between the M_r 80,000 antigen and EBV infection could be demonstrated directly by using sequential serum samples collected in a previous study (5) from acute IM patients. Sera collected immediately after diagnosis and for variable intervals thereafter were both anti-RANA and anti-EBNA negative. However, during the subsequent months, these antibodies either appeared concurrently or one reactivity slightly preceded the other, and titers continued to rise thereafter. In sequential serum samples from two such patients reactivity with the M_r 80,000 antigen was detectable only after both the anti-EBNA and anti-RANA had appeared, but it more closely paralleled the latter (Fig. 3).

Relationship of the M_r 80,000 Polypeptide to EBNA and RANA. The results presented thus far are consistent with the M_r 80,000 antigen representing either EBNA or RANA. In a previous study, cells from a human EBV-infected lymphoblast line (Daudi) were fused with mouse fibroblasts, and RANA and EBNA, as monitored by IF and ACIF, were found to distribute separately in different hybrid clones (11). One RANA$^+$ EBNA$^-$ and two RANA$^-$ EBNA$^-$ lines were available from these hybrids. The immunoblot was positive with the RANA$^+$ EBNA$^-$ extract but was negative with both of the other two hybrid lines (Fig. 4). However, this suggestion that the M_r 80,000 polypeptide represents RANA and not EBNA is tempered by the fact that in subsequent continuous culture this RANA$^+$ EBNA$^-$ subclone ceased expression of both antigens and thus is lost to further investigation. Therefore, an experiment to determine whether the M_r 80,000 peptide is contained in RANA–anti-RANA precipitates was performed. Typical precipitin lines were formed in agarose, thoroughly washed, dissolved, and assayed by the immunoblot procedure along with control agarose that had had antigen without antibody in it. A strong immunoreactive M_r 80,000 polypeptide could be detected in the precipitin product but not in the control (data not shown).

Among the properties reported for EBNA and utilized in previous purification schemes were its unusual heat stability and DNA binding ability (12, 13), either or both of which might afford sufficient enrichment for immunoblot detection. Small aliquots of WI-L2 nuclear extracts were heated to various temperatures above 65°C for 10 min; the precipitated and soluble proteins were separated and equal proportions of the pellet and supernatant were immunoblotted. Whereas ≈90% of the total protein was contained in the 75°C pellet, the majority of the M_r 80,000 polypeptide remained soluble (Fig. 5 *Left*). An enrichment for DNA binding proteins was made by passing a WI-L2 nuclear extract through a small DNA-cellulose column and eluting specifically bound material with 0.4 M NaCl, followed by 1 M NaCl and 6 M urea to strip any protein bound in a more nonspecific manner. Portions of all fractions were again assayed by immunoblotting. The M_r 80,000 polypeptide was absorbed by the DNA-cellulose and eluted with 0.4 M NaCl (Fig. 5 *Right*). Neither the heat-supernatant nor the 0.4 M NaCl eluate from DNA-cellulose exhibited any immunoreactive species of the size (M_r 48,000) previously reported for EBNA that had been purified incorporating these two steps.

Thus, the M_r 80,000 antigen, which appeared to represent RANA, has properties of DNA binding and heat stability like EBNA. This coincidence, together with the failure to detect a second antigen under conditions reported to enrich for EBNA, prompted us to ask whether the antibodies binding the M_r 80,000 polypeptide were capable of fixing complement, because reported assays for EBNA involve complement fixation. Blots of a heat-fractionated WI-L2 nuclear extract were cut into strips and half was probed with individual serum in the customary manner and scored with iodinated anti-human IgG. The second half was reacted with the same series of sera but containing

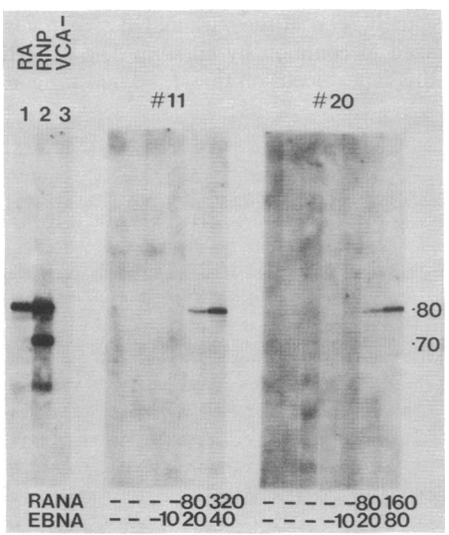

FIG. 3. Sequential serum samples from two acute IM patients reacted with WI-L2 nuclear extracts. The IM sera were numbers 11 and 20 from a previous study (5), collected at various intervals after diagnosis, and were reacted individually with strips bearing the same electroblotted WI-L2 antigen. The anti-EBNA (by ACIF) and anti-RANA (by IF) titers are indicated. Parallel control strips were reacted with RA serum (lane 1), SLE anti-(U1) RNP serum (lane 2), and serum from a VCA$^-$ normal (lane 3). Molecular weights are shown as $M_r \times 10^{-3}$.

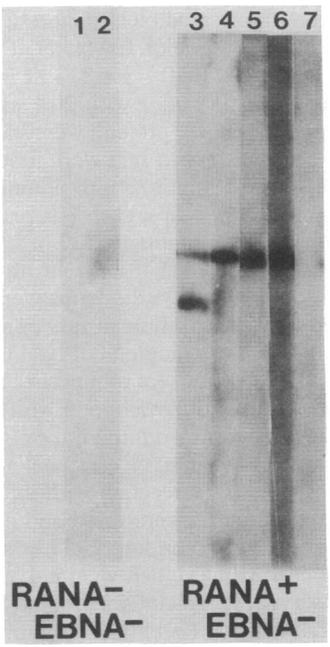

FIG. 4. Nuclear extracts from somatic cell hybrid clones reacted with VCA$^+$ sera. Nuclear extracts were prepared from three existing subclones derived from the fusion of EBV-infected human B lymphoblasts with mouse fibroblasts (10). Equivalent amounts of proteins from the three extracts were resolved on 7.5% acrylamide/NaDodSO$_4$ gels and were transferred to nitrocellulose. Two RANA$^-$ EBNA$^-$ lines were loaded in lanes 1 and 2, and lanes 3–7 contained proteins from the RANA$^+$ EBNA$^-$ line 43.2. The blotted proteins were reacted with the following VCA$^+$ sera: RA serum (lanes 1, 2, and 4); SLE (anti-RNP) serum (lane 3); a second RA serum (lane 5); anti-RANA$^+$ normal serum (lane 6); and anti-RANA$^-$/EBNA$^-$ serum from a patient with early IM (lane 7).

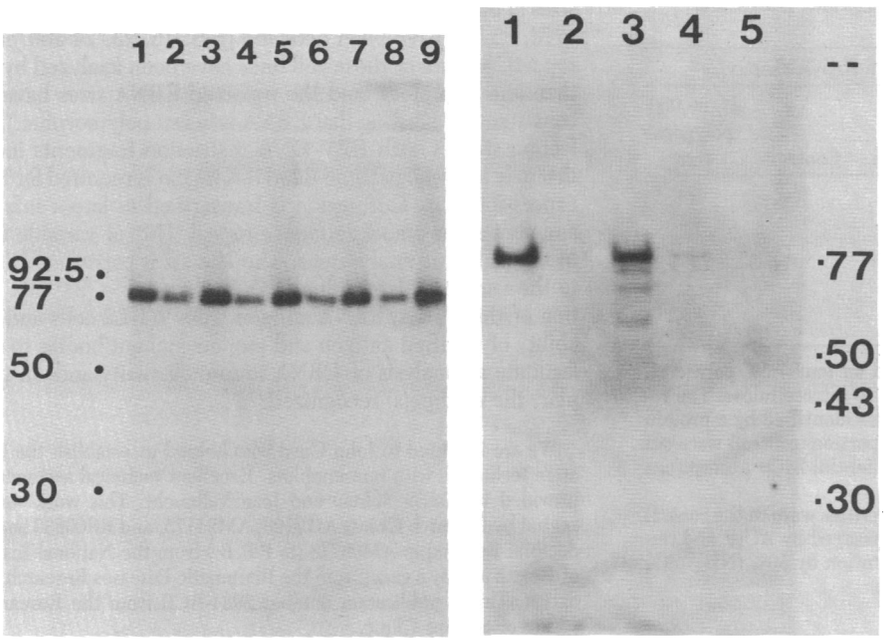

FIG. 5. The M_r 80,000 antigen in WI-L2 cells is a heat-stable DNA binding protein. (*Left*) Heat stability was assayed by immunoblotting equivalent proportions of insoluble (pellet) and soluble (supernatant) fractions from a WI-L2 nuclear extract held at various temperatures for 10 min before centrifuging. Lane 1, control kept on ice; lanes 2 and 3, pellet and supernatant, respectively, from a 65°C treatment; lanes 4 and 5, 75°C; lanes 6 and 7, 80°C; and lanes 8 and 9, 90°C. (*Right*) DNA binding was assayed by passing a WI-L2 extract through a DNA-cellulose column. Portions of each fraction were assayed by immunoblotting with RA serum. Lane 1, the starting nuclear extract; lane 2, nonbound fraction; lane 3, DNA binding protein specifically eluted with 0.4 M NaCl; lane 4, 1 M NaCl eluate; and lane 5, 6 M urea wash. Molecular weights are shown as $M_r \times 10^{-3}$.

VCA$^-$ human sera as a complement source and then was scored with iodinated anti-C4. The results clearly indicated that the antibodies binding to the M_r 80,000 polypeptide were capable of fixing complement (Fig. 6). Thus, the same antigen could be scored by an indirect anti-IgG assay analogous to the indirect IF assay for RANA or by complement fixation analogous to ACIF for EBNA. However, as seen in Fig. 6, there are some sera that react with the M_r 80,000 polypeptide in the direct immunoblot but exhibit little or no reactivity when scored with anti-C4.

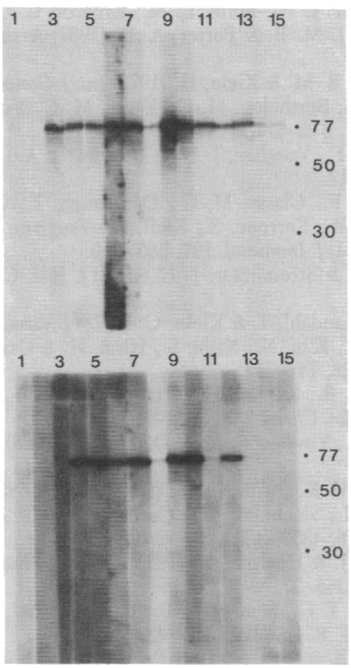

FIG. 6. The M_r 80,000 polypeptide fixes complement. A 75°C heat-treated WI-L2 nuclear extract was electroblotted from two equivalent $NaDodSO_4$ gels. Two strips were reacted in parallel with each serum either in the usual manner (*Upper*) probing with iodinated rabbit anti-human IgG or in the buffer containing complement followed by iodinated anti-C4 (*Lower*). Lane 1, VCA$^-$ serum that was used as a complement source; lane 2, another VCA$^-$; lane 3, VCA$^+$ with 20 mM EDTA to prevent complement fixation; and lanes 4–15, 12 different VCA$^+$ sera. Molecular weights are shown as $M_r \times 10^{-3}$.

To assess the possible relationship of EBNA more directly, the M_r 80,000 band from heat-fractionated extracts was cut from blots as a horizontal strip from several preparative gels together with a control strip from a region not containing reactive polypeptides, and the two were incubated in parallel with RA serum. The M_r 80,000 absorbed and control absorbed antibodies were concentrated by 50% saturated $(NH_4)_2SO_4$ and titered for residual anti-EBNA by ACIF on WI-L2. Anti-EBNA activity was significantly absorbed by the M_r 80,000 polypeptide, giving a three-tube dilution difference in end-point titer from the control (Table 1). Anti-VCA antibody titers dropped only one tube. Because RANA was originally identified based on a precipitin reaction, an assay for anti-RANA precipitating activity was also performed. Faint anti-RANA precipitation lines were seen in double-diffusion at dilutions of 1:2 and 1:4 of the concentrated control serum but no precipitation was seen with the M_r 80,000 polypeptide absorbed serum.

DISCUSSION

Here we report that a single M_r 80,000 polypeptide was reactive in the EBV$^+$ nonproducer B-lymphoblast cell line WI-L2, when assayed by the immunoblot technique using >20 anti-RANA/anti-EBNA$^+$ sera. The antigen clearly is EBV-associated in that it is not detectable in HeLa cells or in the EBV$^-$ B-lymphocyte lines BJAB and Ramos, and antibodies to the antigen are absent from VCA$^-$ sera but arise after EBV infection.

In our initial efforts to determine whether the M_r 80,000 polypeptide represents EBNA or RANA, both of which are expressed in nonproducer EBV$^+$ B lymphoblasts, we investigated whether the M_r 80,000 antigen was detectable in a RANA$^+$ EBNA$^-$ human B-lymphoblast–mouse fibroblast hybrid line. The finding was positive. We also found anti-M_r 80,000 to arise more closely with anti-RANA than with anti-EBNA after EBV infection. These findings suggested that the M_r 80,000 antigen represented RANA. We further showed that RANA precipitin bands redissolved from agarose plates yielded the M_r 80,000 band in the immunoblot analysis. On the other hand, in additional studies, the M_r 80,000 antigen proved to be a heat-stable DNA binding protein, as reported for EBNA; when a complement fixation assay was used in the immunoblot, the anti-

Table 1. The M_r 80,000 polypeptide absorbs anti-EBNA and anti-RANA reactivity

Anti-EBNA reactivity*			Anti-RANA reactivity*		
Dilution	Control	M_r 80,000 polypeptide	Dilution	Control	M_r 80,000 polypeptide
1:8	++	+++	1:2	+	−
1:16	++	+++	1:4	+	−
1:32	+++	++			
1:64	+++	+			
1:128	++	−			
1:256	++	−			

A heated WI-L2 extract was fractionated on four 7.5% polyacrylamide/NaDodSO$_4$ gels and was transferred to nitrocellulose. The region of anti-RANA reactivity (M_r 80,000) was identified by a protein blot. The M_r 80,000 band and a nonreactive portion (control) were cut from each of the four filters and used in sequential 24-hr absorptions of RA serum with anti-EBNA reactivity.

* Residual anti-EBNA and anti-RANA reactivities were in the control and M_r 80,000 polypeptide absorbed sera assayed by ACIF and immunodiffusion, respectively, after concentration by 50% $(NH_4)_2SO_4$ precipitation.

M_r 80,000 antibodies were shown to fix complement like anti-EBNA. Finally, the M_r 80,000 antigen absorbed both antibodies from an anti-RANA/anti-EBNA serum.

If EBNA and RANA truly represent the same EBV-associated antigen, how does one account for the previous evidence for their distinction? The initial distinction (3) was made on the basis of gross discrepancies in titer of anti-RANA and anti-EBNA within given sera. This may conceivably have been on the basis of relative differences in abilities of anti-EBNA/RANA antibodies in given sera to fix complement, and we have seen striking examples of such differences in our studies of the M_r 80,000 antigen in the immunoblot using anti-C4 or anti-Ig sera as the probes (see Fig. 6). A further distinction between EBNA and RANA was made in the reports by Slovin *et al.* (10, 11), who noted differences in staining reactions in cells at various stages in the cell cycle and in somatic cell hybrids. However, as noted in these reports, the staining patterns represent only *expressions* of antigen; thus, for instance, the two staining patterns could have represented (*i*) two antigens, (*ii*) one antigen in two different states of conformation or of complex with other cellular macromolecules, (*iii*) differences in the specific epitopes to which the antibodies in different sera were directed, or (*iv*) differences in concentration or distribution of antigen within a cell with the different methods of tissue fixation used. A recent report by Spelsberg *et al.* (20) described two forms of EBNA (I and II) based upon differences in their association with chromatin. Conceivably, anti-RANA and anti-EBNA may be related to this difference. Anti-RANA cannot be directed to the M_r 82,000 antigen recently described by Hennessy and Kieff (21) because the sera used for the studies described here do not recognize the M_r 82,000 antigen (results not shown). The present findings are consistent with the observations of Catalano *et al.* (5, 6), who noted a similarity in the kinetics of development of anti-RANA and anti-EBNA during the convalescent phase of IM and a significant correlation between titers of the two antibodies in normal adults, and with the observations of Cohen and Lenoir (22), who found significant correlations of anti-RANA with anti-EBNA titers in a number of pathological sera.

The M_r 80,000 EBNA found by directly immunoblotting WI-L2 extracts is considerably larger than that reported in other EBV$^+$ cell types (refs. 12, 13, 15, 23, 24 and present report). However, by using rapid immunoblotting procedures similar or identical to those we used in WI-L2 cells to analyze the EBNA sizes in other EBV$^+$ cells, EBNAs ranging in size from M_r 65,000 to M_r 85,000 have been detected (refs. 15, 23, 24 and present report). Several of these cell lines have been analyzed by more than one laboratory, and the reported EBNA sizes have been consistent, suggesting that EBNA is in fact polymorphic. Transfection studies with EBV DNA restriction fragments indicate that only a 1.9-megadalton *Bam*HI *K* region is required for EBNA expression (25). This region is transcribed in latent infections and contains a simple sequence repeat (IR3) of variable length in the different viral strains. The IR3 sizes correspond closely to the various polymorphic EBNAs detectable (24, 26). Isolation of the EBNA/RANA antigen from WI-L2 cells and availability of purified antigen and monoclonal antibodies to it will facilitate an analysis of EBNA immunogenicity and, in particular, the epitope(s) recognized.

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1. Alspaugh, M. A. & Tan, E. M. (1975) *J. Clin. Invest.* **55**, 1067–1073.
2. Alspaugh, M. A. & Tan, E. M. (1976) *Arthritis Rheum.* **19**, 711–719.
3. Alspaugh, M. A., Jensen, F. C., Rabin, H. & Tan, E. M. (1978) *J. Exp. Med.* **147**, 1018–1027.
4. Catalano, M. A., Carson, D. A., Slovin, S. F., Richman, D. D. & Vaughan, J. H. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 5825–5828.
5. Catalano, M. A., Carson, D. A., Niederman, J. C., Feorino, P. & Vaughan, J. H. (1980) *J. Clin. Invest.* **65**, 1238–1242.
6. Catalano, M. A., Carson, D. A., Stastny, P., Freer, S. & Vaughan, J. H. (1980) *Arthritis Rheum.* **23**, 1049–1052.
7. Venables, P. J. W., Roffe, L. M., Erhardt, C. C., Maini, R. N., Edwards, J. M. B. & Porter, A. B. (1981) *Arthritis Rheum.* **24**, 1459–1464.
8. Reedman, B. M. & Klein, G. (1973) *Int. J. Cancer* **11**, 499–520.
9. Lenoir, G., Berthelon, M.-C., Favre, M.-C. & de Thé, G. (1976) *J. Virol.* **17**, 672–674.
10. Slovin, S. F., Vaughan, J. H. & Carson, D. A. (1980) *Int. J. Cancer* **26**, 9–12.
11. Slovin, S. F., Glassy, M. C., Dambaugh, T., Catalano, M. A., Curry, R. A., Ferrone, S., Kieff, E., Vaughan, J. H. & Carson, D. A. (1981) *J. Immunol.* **127**, 585–590.
12. Baron, D. & Strominger, J. L. (1978) *J. Biol. Chem.* **253**, 2875–2881.
13. Luka, J., Lindahl, T. & Klein, G. (1978) *J. Virol.* **27**, 604–611.
14. Matsuo, T., Kibi, N., Nishi, S., Hirai, H. & Osato, T. (1978) *Int. J. Cancer* **22**, 747–752.
15. Sculley, T. B., Kreofsky, T., Pearson, G. R. & Spelsberg, T. C. (1983) *J. Biol. Chem.* **258**, 3974–3982.
16. Levy, J. A., Virolainen, M. & Defendi, V. (1968) *Cancer* **22**, 517–524.
17. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
18. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76**, 4350–4354.
19. White, P. J. & Hoch, S. O. (1981) *Biochem. Biophys. Res. Commun.* **102**, 365–371.
20. Spelsberg, T. C., Sculley, T. B., Pikler, G. M., Gilbert, J. A. & Pearson, G. R. (1982) *J. Virol.* **43**, 555–565.
21. Hennessy, K. & Kieff, E. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 5665–5669.
22. Cohen, J. H. M. & Lenoir, J. M. (1983) *Biomedicine* **36**, 246–249.
23. Strnad, B. C., Schuster, T. C., Hopkins, R. F., III, Neubauer, R. H. & Rabin, H. (1981) *J. Virol.* **38**, 996–1004.
24. Hennessy, K., Heller, M., van Santen, V. & Kieff, E. (1983) *Science* **220**, 1397–1398.
25. Summers, W. P., Grogan, E. A., Shedd, D., Robert, M., Lui, C.-R. & Miller, G. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 5688–5692.
26. Heller, M., van Santen, V. & Kieff, E. (1982) *J. Virol.* **44**, 311–320.