

AUTOANTIBODIES IN INFECTIOUS MONONUCLEOSIS  
HAVE SPECIFICITY FOR THE GLYCINE-ALANINE  
REPEATING REGION OF THE EPSTEIN-BARR VIRUS  
NUCLEAR ANTIGEN

By GARY RHODES\*, HELMUT RUMPOLD\*<sup>‡</sup>, PEKKA KURKI\*<sup>§</sup>,  
KEVIN M. PATRICK<sup>1</sup>, DENNIS A. CARSON\*, AND JOHN H. VAUGHAN\*

*From the \*Department of Basic and Clinical Research, Scripps Clinic and Research  
Foundation, La Jolla, California 92037; the <sup>‡</sup>Institute of Clinical Chemistry,  
University of Vienna, A-1090 Vienna, Austria; the <sup>§</sup>Department of Pathology,  
University of Helsinki, SF-00290 Helsinki, Finland; and the <sup>1</sup>Student Health Services,  
San Diego State University, San Diego, California 92182*

The factors responsible for the appearance of autoantibodies after viral infections have not been well understood. Infectious mononucleosis (IM)<sup>1</sup> is characterized by a particularly high incidence of autoantibodies, including the heterophile antibody, rheumatoid factor, and among those assayed by immunofluorescence, antibodies to the cytoskeleton (1-7). 70-90% of acute IM patients have high titers of IgM autoantibodies to the vimentin class of intermediate filaments (4). 30-60% have IgM antikeratin antibodies (5). Individuals who have had Epstein-Barr virus (EBV) infections in the distant past, or individuals with no previous EBV infection, have none or only very low titers of these antibodies. Autoantibodies are also produced during other viral infections (6, 8).

A number of hypotheses have been generated to account for such autoantibody production. The first is that the antibodies are produced in response to normally sequestered cellular proteins that are released during cell lysis and death, thereby allowing them to be taken up by macrophages and presented as antigen to the immune system. A second mechanism proposed is that antibodies made to microbial antigens can crossreact with normal cellular proteins. The best known example of this mechanism is the production of antibodies to the myocardium during streptococcal infection (9, 10). This mechanism has recently been reinvestigated with respect to viral or mycoplasmal antigens (11-16) and popularized under the term molecular mimicry, although perhaps the more neutral term epitope homology would be better. A third mechanism particularly pertinent to infection by EBV is that the virus acts as a polyclonal B cell activator. The virus randomly infects B cells and induces the secretion of whatever antibodies the B

This work was supported in part by grants AM21175-09 and RR00833 to the General Clinical Research Center of Scripps Clinic. Gary Rhodes is supported by training grant AM07144-12 and by a stipend from Johnson & Johnson Inc. Helmut Rumpold is supported by the Max Kade Foundation. This is publication number 4347BCR from the Research Institute of Scripps Clinic.

<sup>1</sup> *Abbreviations used in this paper:* EBNA, Epstein-Barr nuclear antigen; IM, infectious mononucleosis; PM buffer, powdered milk buffer.

```

1  MSDEGPGTGP  GNGLGEKGD  T  SGPEGSGGSG  PQRREGDNHG  RGRGRGRGRG
51  GRRPGAPGGS  GSGPRHRDGV  RRPQKRPSCI  GCKGTHGGTG  AGAGAGGAGA
101 GGAGAGGGAG  AGGGAGGAGG  AGGAGAGGGA  GAGGGAGGAG  GAGAGGGAGA
151 GGGAGGAGAG  GGAGGAGGAG  AGGGAGAGGG  AGGAGAGGGA  GGAGGAGAGG
201 GAGAGGAGGA  GGAGAGGAGA  GGGAGGAGGA  GAGGAGAGGA  GAGGAGAGGA
251 GGAGAGGAGG  AGAGGAGGAG  AGGGAGGAGA  GGGAGGAGAG  GAGGAGAGGA
301 GGAGAGGAGG  AGAGGGAGAG  GAGAGGGGGR  RGGSGGRGRG  GSGGRGRGGS
351 GRRRGRGRER  ARGGSRERAR  GRGRGRGEKR  PRSPSSQSSS  SGSPRRRPPP
401 GRRPFFHPVG  EADYFEYHQE  GGPDGEPDVP  PGAIEQQPAD  DPGEGPSTGP
451 RGQGDGGRRK  KGGWFGKHRG  QGGSNPKFEN  IAEGLRALLA  RSHVERTTDE
501 GTWVAGVFVY  GSKTSLYNL  RRGTAIAIPQ  CRLTPLSRLP  FGMAPGPGPQ
551 PGPLRESIVC  YFMVFLQTHI  FAEVLKDAIK  DLVMTKPAPT  CNIRVTVCSE
601 DDGVDLPPWF  PPMVEGAAAE  GDDGDDGDEG  GDGDEGEEGQ  E*

```

FIGURE 1. Peptide sequence of the EBNA-1 molecule (one-letter amino acid code). The glycine-alanine repeat is shaded. Peptide F12 begins at residue 1 and peptide F13 at residue 54, both entirely outside the glycine-alanine repeat. P27 begins at residue 83 and spans the NH<sub>2</sub>-terminal end of the repeat. P60 begins at residue 314 and ends at the R terminating the repeat. F14, F15, F16, and P62 occur four, three, one, and three times, respectively, entirely within the repeat.

cell is preprogrammed to make, antibodies generally unrelated to the virus itself. Some of these antibodies may include autoantibodies (17–20).

Among the many virally encoded proteins generated during EBV infection, the Epstein-Barr nuclear antigen (EBNA-1) has been of particular interest. It is generated in latently as well as productively infected B cells, and antibodies to it arise after infection and persist for the rest of the individual's life (21, 22). The EBNA-1 molecule plays a critical role in maintenance of viral DNA in the infected cell (23). In addition, patients with certain autoimmune diseases have higher levels of antibodies to EBNA-1 (24, 25) and to synthetic peptides derived from EBNA-1 (26) than a normal control group.

The EBNA-1 protein has a very unusual structure in which about one-third of the protein consists of a repeated sequence that contains only glycine or alanine (27–29) (Fig. 1). DNA sequences homologous to the viral region that encodes the glycine-alanine portion are present on all human chromosomes except the Y (30). These sequences are transcribed into a number of RNA molecules (31–33) and some may be translated into protein (33, 34).

We have examined the IgM antibodies produced during acute IM, and have found antibodies that recognize at least 10 cellular proteins. Most of the autoantibodies reactive with these proteins are highly crossreactive with each other and are inhibited by synthetic peptides containing portions of the glycine-alanine repeating area of the virally encoded EBNA-1 protein. Thus the IgM autoantibodies produced during IM appear to be made to a specific region of a viral protein, and crossreact with homologous epitopes on host proteins. The IgG anti-EBNA-1 antibodies that arise during late convalescence fail to exhibit the crossreactive features of the IgM anti-EBNA-1.

### Materials and Methods

*Cells.* Cell lines were obtained from the American Type Culture Collection (Rockville, MD), except for the line DHL-9 (35, 36). They were grown in RPMI 1640 supplemented with 10% FCS and 1% L-glutamine. The cells were harvested in late exponential growth and washed four times with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 6.8). 100  $\mu$ l of diluted electrophoresis mix (2% SDS, 2% 2-ME, 0.004% bromphenol blue, 40 mM Tris-HCl, pH 6.8, and 15% glycerol) and 2  $\mu$ l of PMSF (5 mM PMSF dissolved in ethanol) were added for each  $10^6$  cell and the solution was heated to 100°C for 5 min and stored frozen. Protein concentration was determined by the Peterson modification (37–39) of the Lowry method (40). This procedure results in a protein concentration of 1–2 mg/ml.

*Cell Fractionation.* Cells were lysed and nuclei were pelleted as described (41). The nuclear pellet was resuspended in DEM, boiled, and stored at –20°C. This we called the nuclear fraction. The supernatant from the above step was spun at 145,000 *g* for 1 h (sufficient to pellet all particles having a sedimentation coefficient >120 S). The supernatant from this spin was designated the cytoplasmic fraction. It was mixed with one-fifth the volume of 5 $\times$  DEM, boiled, and stored frozen until needed. The pellet was resuspended in DEM and called the particulate fraction. It contains cell organelles as well as membranes. Because of the way the supernatant was removed, the membrane fraction is slightly contaminated with the cytoplasmic fraction.

*Sera.* Serum samples from IM patients were collected from students when they presented with clinical symptoms, and again 1 mo later. The initial serum samples were all heterophile-positive and all had elevated IgM and low or no IgG anti-peptide P62 antibody levels (42). 30–50 ml of blood was collected directly in 50 ml of RPMI-1640 tissue culture media, and this was then loaded onto a Ficoll density gradient to isolate cells. After centrifugation the serum supernatant was removed and frozen. Thus, the serum samples are diluted 1:2–1:2.5 when frozen. All dilutions referred to in this paper refer to dilutions from this frozen sample.

*Peptides and Proteins.* The peptides used in this study were synthesized by the solid-phase method of Merrifield (43), modified as described (44). The sequences of the peptide are: F12, IMSDEPGTGPNGGLGE; F13, PGAPGGSGSGP; F14, GGAGGAGGAGGGGAG; F15, GAGGAGAGGGAGGAGG; F16, GGAGAGGAGAGGAGAGGAGAG; P27, KGTMGGTGAGAGAGGAGAG; P60, GGGAGAGGAGAGGGGR; P62, AGAGGGAGGAGAGGGAGGAG. They represent multiple sequences in the NH<sub>3</sub>-terminal half of the EBNA-1 molecule, within which the entire glycine-alanine repeat resides (Fig. 1). All peptides have an additional COOH-terminal cysteine that is not present in the EBNA-1 sequence, but was added by us to facilitate conjugating them to protein carriers. Purified keratin was obtained from Sigma Chemical Co. (St. Louis, MO).

*Immunoblotting.* We mixed FITC-labeled marker proteins with 500–700  $\mu$ g of protein extract, and this was loaded onto one 10-cm-wide slot of a 7.5% acrylamide gel. The gel was run and transferred to nitrocellulose by the Billings et al. (25) modification of the Towbin et al. (45) method. The sheet was stored at –20°C until use. We cut 0.5-cm strips from the sheet and placed them in powdered milk (PM) buffer (3% commercial powdered milk dissolved in BBS: 10 mM borate, 150 mM NaCl, pH 8.3) for 1 h. Patients' sera were diluted 1:20 or 1:50 in PM and reacted with the strip for 1 h at room temperature. The strips were then washed with BBS and reacted with a solution of 0.6  $\mu$ g/ml (in PM) of affinity-purified rabbit anti-human IgM (The Jackson Laboratories, Avondale, PA;  $\mu$  chain-specific) for 1 h at room temperature. After washing with BBS, the strips were reacted for an additional hour at room temperature with a detecting solution of iodinated goat anti-rabbit IgG antibody. This solution was made by labeling commercial antibody (Kirkegaard and Perry, Gaithersburg, MD; affinity purified) by the chloramine T method (46). The iodinated antibody was diluted in PM and used at an isotopic concentration of  $2 \times 10^5$  cpm/ml (sp act  $\sim$ 1  $\mu$ Ci/ $\mu$ g). The strips were then washed and dried, and the bands were detected by autoradiography as previously described (25, 42).

Peptide inhibitions were done by diluting the sera 1:50 in PM and adding peptide to a final concentration of 500  $\mu$ g/ml. This solution was incubated overnight at 4°C and then used to blot as described above.

**Antibody Elution.** We cut a 3.0-cm-wide segment from a nitrocellulose strip that had a Wi-L2 extract transferred to it. This was reacted with an IM patient's serum diluted 1:20 in PM for 2 h at room temperature. Two strips 0.4-cm wide were then cut from each end and these were washed and treated with rabbit anti-human IgM as described above, while the remaining 2.2-cm strip was returned to the patient's serum. After 1 h at room temperature the smaller strips were washed and incubated for 1 h with a 1:400 dilution of alkaline phosphatase-coupled affinity-purified goat anti-human IgM serum (Boehringer Mannheim Biochemicals, San Diego, CA). The bands were then detected with a color reaction that is a modification of a histological staining method for alkaline phosphatase (47). The strips were placed in 5 ml of 50 mM borate buffer, pH 9.5, containing 0.1 ml of a 10 mg/ml solution of fast violet and 75  $\mu$ l of Naphthol AS-BI phosphate (30 mg/ml solution dissolved in *N,N*-dimethylformamide). Both reagents are from Sigma Chemical Co. A recent paper by Luka et al. (34) also used a similar procedure. After 10 min in the substrate solution, the reaction was stopped by washing with PBS and then water.

The larger 2.2-cm strip was now removed from the serum, washed with BBS and aligned with the smaller strips. Horizontal pieces were cut in the larger strip, which correspond to the visible bands. These were placed in plastic conical centrifuge tubes and 1.0 ml of 50 mM diethylamine, 150 mM NaCl, pH 11.5, was added. The solution was gently mixed at room temperature and the strip was withdrawn. The solution was neutralized with 0.2 M phosphate buffer, pH 2.0, and BSA was added to a final concentration of 200  $\mu$ g/ml. The solution is diluted 1:1 in 2 $\times$  concentrated PM and probed with a new strip as described above.

## Results

*Sera of Acute IM Patients Have IgM Antibodies to a Number of Antigens Present in EBV-transformed B Cells.* This phenomenon is shown in Fig. 2, *left*, where we have blotted the sera of nine acute IM patients on electrophoresed extracts of CA66, an EBV-transformed B cell line. A strikingly similar spectrum of antigens is seen with all nine sera, each of which recognizes more than a dozen bands. The nine strongest antigens, which are most evident in Fig. 2, range in size from 55 to 120 kD. These sera were all routine samples taken from patients when they first presented with clinical symptoms. The same bands, or a subset of them, occur in all of the several dozen acute IM sera we have screened for IgM antibodies. The antibodies are not present in sera of two IM patients drawn before infection (our unpublished data). They appear shortly after the first day of illness, reaching a peak in 2–4 wk and then gradually declining over the next year (48).

The IgM antibody pattern seen in IM patients contrasts sharply with that seen in normal individuals. IgM antibodies to these antigens are absent in normal healthy adults, both viral capsid antigen (VCA)-positive and VCA<sup>-</sup>. The acute IM patients have no IgG antibodies to any protein in an EBV-transformed B cell extract, whereas normal individuals with past EBV infection have IgG antibodies to a single protein, the EBNA-1 protein (Fig. 2 *right*) (25, 49). Together these data suggest that the antibodies seen in the IM patients are a direct response to the acute viral infection.

All nine of the IM sera shown in Fig. 2 react also with purified human epidermal keratin (data not shown). This protein appears on blots as two fuzzy bands with molecular masses of 65 and 58 kD. These bands are indistinguishable from 65 and 58 kD bands seen in blots with the cell extracts (Figs. 2–6). The antikeratin reaction is extremely sensitive and can detect <25 ng of protein in a

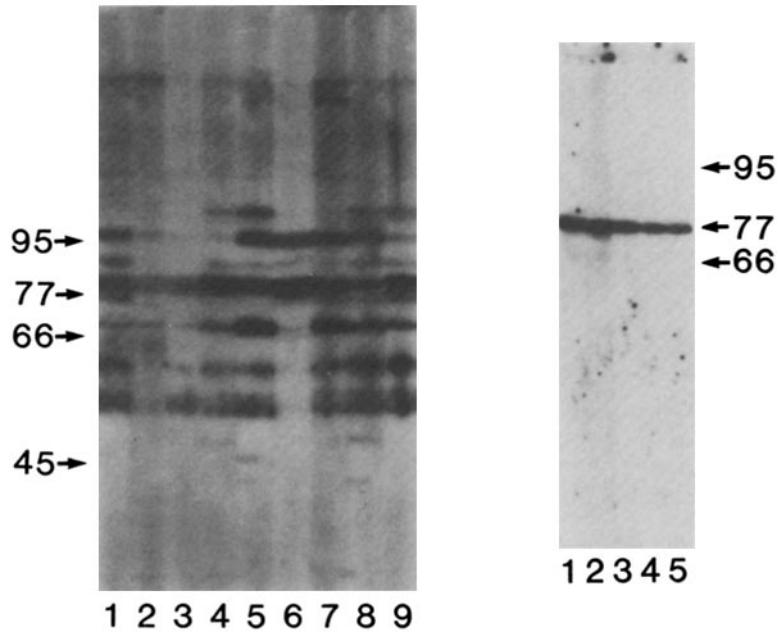


FIGURE 2. Immunoblots of sera from acute IM and normal individuals. (*left*) The IM sera were diluted 1:20 and used to probe strips of an electrophoresed extract of the EBV<sup>+</sup> CA66 cell line. The bands were developed with a rabbit anti-human IgM. Normal sera show no bands under these conditions. (*right*) Sera from five normal individuals were diluted 1:100 and used to blot similar extracts. IgG antibodies were detected. Molecular mass (kD) is shown at sides.

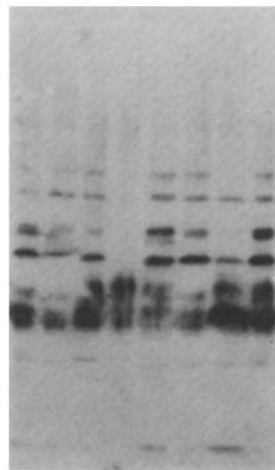


FIGURE 3. Acute IM serum blotted on strips of electrophoresed extracts of a variety of cells. The cells used to make the extracts were: 1, Wi-L2 (EBV<sup>+</sup> B cells); 2, MC116 (EBV<sup>+</sup> B cells); 3, HeLa (EBV<sup>-</sup> epithelial cell line); 4, GM2504 (EBV<sup>-</sup> fibroblasts); 5, B95-8 (EBV<sup>+</sup> producer marmoset B cell); 6, EW66 (EBV<sup>+</sup> B cells); 7, human thymocytes (EBV<sup>-</sup>); 8, K562 (EBV<sup>-</sup> erythroleukemic). Serum 5 (Fig. 1) was diluted 1:20 and used in these blots. 16  $\mu$ g of protein was loaded in each lane. Molecular mass (kD) is shown at right.

1 2 3 4 5 6 7 8

lane of a gel. Keratin is not made in any of the cells we used and probably arises through contamination of buffers with fragments of human skin (50). The amount of contamination varies with the extract (compare Fig. 2 with Fig. 3) and produces the only variation in the banding pattern.

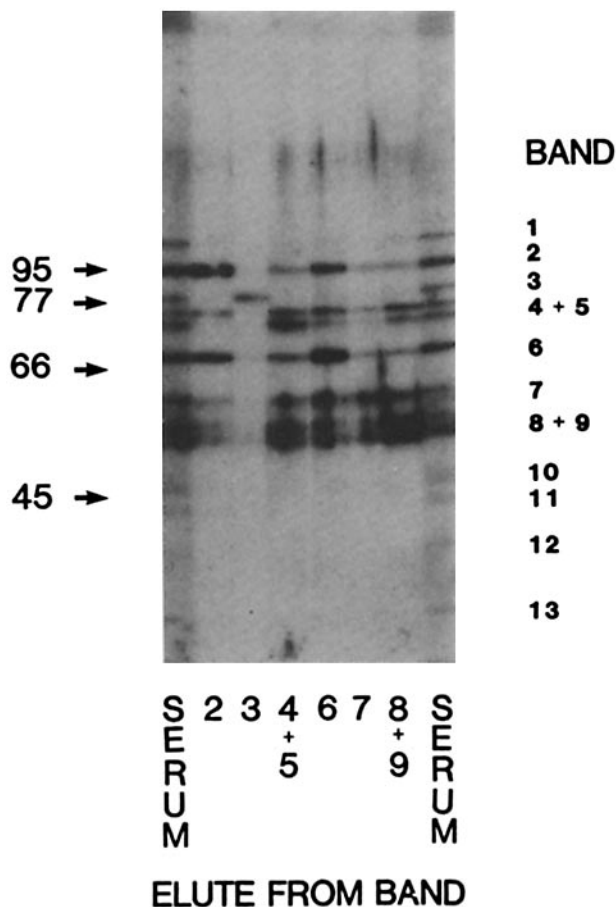


FIGURE 4. Elution and re-probing with antibodies from an IM serum. Serum 5 (Fig. 1) was used for preliminary binding to strips of electrophoresed WI-L2 cell extracts (left- and right-most lanes; marked *SERUM*). Antibodies were eluted from bands 2, 3, 4 + 5, 6, 7, and 8 + 9 (labeled on the vertical axis), and then used as probes on new strips of electrophoresed WI-L2 cell extracts identical to those from which the eluates were made. The horizontal axis is labeled by the band from which the antibody is derived. The eluted antibodies from all but band 3 were highly cross-reactive. Molecular mass (kD) is shown at left.

We chose one of the sera (serum 5 in Fig. 2) as the prototype with which to do further studies. Fig. 3 shows blots obtained when this serum was used to probe extracts from a number of different cells. Lanes 1, 2, 5, and 6 are extracts of various EBV-infected B cell lines. The rest are from EBV<sup>-</sup> cell lines. One can see the same pattern of bands in all extracts (except lane 4) whether the cells contain the EBV genome or not. Thus, numerous antigens recognized by the IgM antibodies in acute IM sera are host-encoded proteins; they are autoantigens.

*IgM Autoantibodies Are Crossreactive.* Since autoantibodies to the same cellular proteins are present in almost all acute IM sera, we looked for a relationship between them. We performed a standard immunoblot on the WI-L2 extract; then we cut out the various bands and eluted the antibodies from them. These antibodies were then used to reprobe other strips from the same cellular extract. The results are shown in Fig. 4. The most prominent bands are labeled 1–9 and are shown at the side in the figure. The source of the antibody probe is shown at the bottom. Antibodies eluted from band 3 bound only to the same band 3. In contrast, antibodies eluted from most of the other bands reacted not only with their own bands, but also with all others except bands 1 and 3. Most of the antigens detected are crossreactive, and all are host encoded except for band 4,

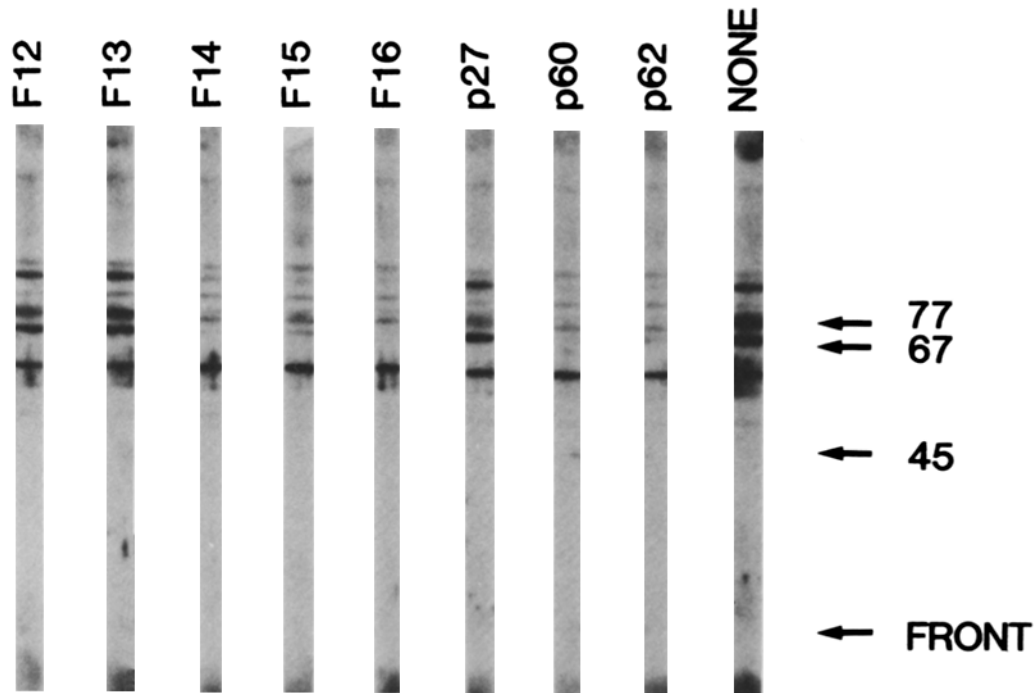


FIGURE 5. Inhibition of IgM autoantibodies by synthetic peptides. The peptides are derived from various portions of the EBNA-1 sequence (29). Peptides F12 and F13 are NH<sub>2</sub>-terminal to the glycine-alanine repeat, peptide P27 is in the transition region, and the other peptides are from various portions within the glycine-alanine region. Serum 5 was diluted 1:50 and the peptides were added to a final concentration of 500  $\mu$ g/ml and incubated overnight before blotting. Molecular mass (kD) is shown at right.

which contains the virally encoded EBNA-1 protein as well as a host antigen. Thus there is one viral and a variety of cellular proteins that have a common or closely related epitope.

*Autoantibodies Are Inhibited by Glycine-Alanine Peptides.* We have shown separately that the glycine-alanine portion of the EBNA-1 protein is the major epitope for IgG anti-EBNA-1 antibodies (51). We asked if the shared epitope seen by the IgM antibodies in IM was related to this same repeating structure. A number of peptides with sequences corresponding to various portions of the EBNA-1 molecule were used as inhibitors in the immunoblotting procedure. The data are shown in Fig. 5. These experiments were done at a higher serum dilution than those of Fig. 4, so that only the most prominent bands of the previous figure, corresponding to bands 2, 4 + 5, 6, and 7-9, are easily seen.

The peptides F12 and F13, which do not contain the glycine-alanine repeating sequence (see Materials and Methods), do not significantly inhibit antibody binding at the concentration of inhibitor used. Peptide P27 also is a weak inhibitor. This peptide crosses the juncture of the repeat and thus contains two regions, one of which is composed of glycine-alanine and the other of which is not. The remaining peptides all contain only glycine-alanine and they all inhibit antibody binding to band 2 (the uppermost dark band) and bands 4-6 (the wide

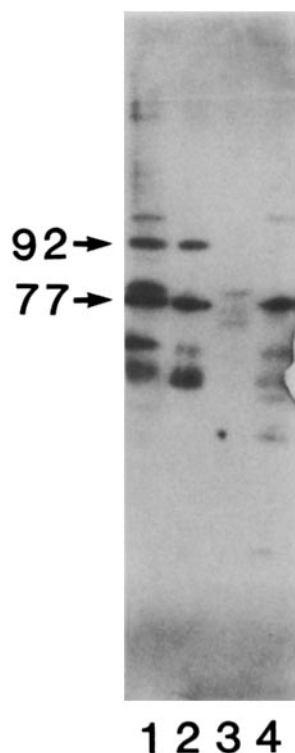


FIGURE 6. Western blot analysis of subcellular fractions. Lane 1 contains an extract from whole cells, 2 from isolated nuclei, 3 from the cytosol, and 4 is from the particulate (membrane and organelle) fraction. The extracts were probed with IM serum 5 diluted 1:20. 30  $\mu$ g of protein was loaded in lane 1, 20  $\mu$ g was loaded in 2 and 4, and 13  $\mu$ g in lane 3. The mobility of molecular mass marker proteins (kD) is indicated on the left side of the figure.

dark bands at 77 kD) and band 7 (strong band at 69 kD). There is also inhibition of the fuzzy keratin bands in the area 55–65 kD, although one prominent antigen in this region is not inhibited at all. Also note that band 1 (weak band just above 2) and band 3 (weak band at 82 kD) are not inhibited by any of the peptides. The same pattern of inhibition is seen with three other sera in the series shown in Fig. 1. The conclusion from these experiments is that antibody binding to most of the crossreacting antigens are inhibited by the glycine-alanine-containing peptides and not by peptides with other sequences. Thus the crossreacting epitope present in the autoantigens is represented in the glycine-alanine region of EBNA-1.

*Cellular Autoantigens Are Distributed Throughout the Cell.* Wi-L2 and K562 cells are fractionated into nuclear, cytoplasmic, and particulate (membrane and organelle) components. Fig. 6 shows a western blot of each of these fractions along with an extract from unfractionated whole cells. Strong bands at 92 and 77 kD are found in the nuclear fraction (Fig. 6, lane 2). Weaker bands at 82, 76, and 72 kD are seen in the cytoplasmic fraction (Fig. 6, lane 3). Some normal individuals have IgG antibodies to the 82 kD protein (H. Rumpold, G. Rhodes, and J. H. Vaughan, manuscript in preparation). The particulate fraction has antigens of 105, 77, 49, and 44 kD. Additional bands at 69 and 55 kD are found in the particulate fraction while a band at 67 kD and a broad band corresponding to a molecular mass of 55–60 kD are seen in the nuclear fraction. Some of the signal in this region is due to contaminating epidermal keratin, but there also seem to be normal cellular antigens in this region. The bands at 92 and 77 kD



are also present in a Triton-insoluble extract of HeLa cells and these may represent components of the cytoskeleton. It is interesting that almost all of the signal seen by immunofluorescence in these sera is directed at the vimentin class of intermediate filaments (1–5, 7). The EBNA-1 protein runs at 77 kD and is found both in the nuclear and cytoplasmic fractions prepared by this method (25). The weak band seen in the cytoplasmic fraction at 77 kD is the EBNA-1 protein (our unpublished data).

*Crossreacting Antigens Are Not Proteolysis Fragments.* We have done several experiments to try to eliminate the possibility that proteolysis generates the series of crossreacting antigens. The extracts are prepared by harvesting the cells, washing them, adding protease inhibitor, and boiling them in SDS. The whole process takes less than 10 min (see Materials and Methods). Very little, if any, breakdown of EBNA-1 is seen with this procedure, as evidenced by the single 78–80 kD EBNA-1 band seen in these extracts with IgG anti-EBNA-1 in normal sera. No change in the pattern is seen if the cells are left at room temperature for 1 h before making the extract. There is also no major change after storage of the extract either overnight at room temperature or for many months at  $-20^{\circ}\text{C}$ . The same pattern of antigens is also seen in a variety of cell lines. Perhaps the best argument against extensive proteolysis is the cellular distribution of the antigens. As discussed above, the antigens are located in separate cellular compartments, making it unlikely that the antigens are all derived from a precursor polypeptide that contains the crossreacting epitope. All of these data, while indirect, suggest that no major proteolysis occurs in the cells or in the extract.

### Discussion

These studies show that there is a series of at least nine and probably more normal cellular proteins that are recognized by IgM antibodies produced during acute and convalescent IM. By definition, these cellular proteins are specified as autoantigens. Some or all of these antigens are present in a variety of EBV-infected and noninfected B cell lines, in an erythroid cell line (K562), in several T cell lines, and in human thymocytes. The antigens are also present in HeLa cells, but only a subset of them is detectable in a human fibroblastic cell line (GM2504). Similar antigens are also present in murine cells and a bovine cell line (our unpublished data), although we have not made a systematic study of species distribution. It thus appears that these antigens are widespread both in cell lineages and among the higher vertebrates.

The human autoantibodies reactive with these antigens display extensive crossreactivity, in that eluates from a given cellular antigen in a Western blot are able to recognize a series of other cellular proteins. Antibody binding to all of these proteins is inhibited by synthetic peptides having sequences from the glycine-alanine region of EBNA. Further, rabbit antibodies made to these same peptides are able to recognize a subset of the crossreactive antigens (our unpublished data).

The amino acid sequences of the crossreacting epitopes recognized by the IM sera apparently need not conform precisely to the glycine-alanine prototype found in EBNA. Human epidermal keratin has no glycine-alanine sequences,

but it does contain a glycine-rich area flanking a polyserine region (. . . GGGYGGGFSSSSSFGSFFGGGYGGGL . . .), which we judge to be the most likely site for the crossreacting epitope on this molecule (52, 53). Some of the other cellular antigens may have peptide sequences more closely related to the viral sequence. Siebl and Wolf (33) were able to select two cellular transcripts that hybridized to an EBV DNA fragment coding for EBNA-1. Translation of the RNA produced proteins of 92 and 84 kD, which may be the same as our 92 and 82 kD bands. The IM sera also recognize a 62 kD protein (sometimes obscured by the keratin bands) that has a mobility identical to a protein described by Luka et al. (34). The 62 kD antigen reacts with a monoclonal antibody made to EBNA-1. Sequence variability in the crossreactive epitope may indicate that secondary structure is an important determinant of the epitope (54 and see below).

It is striking that all of the 42 IM patients we have tested have IgM autoantibodies to most of the same cellular proteins. The amounts of these autoantibodies vary from patient to patient, and some patients' sera may miss some of the weaker bands. However, every patient we have tested does have antibodies to the prominent antigens at 92, 78–82, 69, 62, and 56 kD, although the last two or three are sometimes obscured by contaminating epidermal keratin. The antibodies are absent in the preillness sera, but are present in the first postonset sample of four IM patients for which we have such serial samples. They persist at a slowly decreasing concentration for many months after the disease. Most patients have a small but detectable amount of IgM autoantibodies a year after the disease, and these may persist for up to 2 yr in some people.

The kinetics of appearance of antibody to EBNA-1 is highly unusual among the anti-EBV antibodies. IgG antibodies to all other EBV antigens are present early in the symptomatic phase of the disease (21, 22), which is some weeks after the initial infection. In contrast, IgG anti-EBNA-1 antibodies, as measured by the classical anticomplement immunofluorescence (ACIF) assay (44), usually become detectable only months after infection. This fact is exploited in the diagnosis of primary acute EBV infection (21, 22, 48). We have previously reported (26, 42) that an ELISA assay for anti-EBNA-1 antibodies, using as antigen synthetic peptides from the glycine-alanine region of EBNA, detects IgM anti-EBNA-1 peptide antibodies during the early symptomatic stage of the disease, but IgG anti-EBNA-1 peptide antibodies (as with the ACIF measurement of anti-EBNA-1) usually do not appear for several months.

In contrast to IgM, IgG antibodies to EBNA-1 are very specific for EBNA-1; normally they do not recognize the cellular proteins seen by the IgM anti-EBNA-1 antibodies (25, 49). Both IgG and IgM anti-EBNA-1 antibodies are, nevertheless, directed predominantly to the same glycine-alanine region in EBNA-1. Perhaps the early IgM autoantibodies, produced as part of a T cell-independent anti-EBNA-1 glycine-alanine response, switch to the IgG isotype only with difficulty. This switch must await clonal expansion of rare anti-EBNA-1 antibodies that do not recognize the crossreactive self antigens. The production of IgG anti-EBNA-1 antibodies thus would be the result of a lengthy and time-consuming process and would ordinarily result in antibodies specific for EBNA-1 and not crossreactive with the similar cellular antigens.

EBNA-1 antibody synthesis provides a system to study the mechanism of immune tolerance during a normal human immune response. Here a viral protein that shares epitopes with a number of human components is introduced well after the maturation of the immune system. The early IgM antibodies are broadly crossreactive, while the IgG antibodies react exclusively with the viral protein. Despite the increased specificity of the IgG isotype, both the IgG and IgM antibodies are directed at the same glycine-alanine epitopes (51). These results can be used to exclude several proposed mechanisms of tolerance (reviewed in 55). We see the same autoantibodies occurring in every IM patient we have examined, so tolerance cannot be due to the absence of B cell precursors. The increase in crossreacting antibodies early in the disease also makes it unlikely that direct B cell blocking occurs. The most reasonable assumption is that tolerance involves T cells, perhaps during isotype switching. Further characterization of the system should allow more definitive conclusions to be drawn.

Although our studies do not demonstrate the means by which these glycine-alanine-crossreactive IgM autoantibodies arise, it seems reasonable to regard them as a component of the anti-EBNA-1 response. The kinetics of their appearance and disappearance is consistent with this (26, 42). The alternative explanation that the autoantibodies are simply the result of immunization with cellular antigens spilled from damaged or dying cells may conceivably account for some autoantibodies. However, we do not see anti-glycine-alanine antibodies in the sera of burn or trauma patients (none seen among 20 patients examined), and they are present in <5% (3 of 64) of patients with other acute viral infections (our unpublished data).

Polyclonal B cell activation in its simplest form is also unlikely by itself to account for the autoantibodies we describe here, because of its inherently random nature. If this mechanism were responsible for producing the autoantibodies we describe, one would have expected only a portion of the patients to produce autoantibodies to any specific protein, and one would not expect the autoantibodies to be crossreactive. A possibility not excluded by these data is polyclonal activation of a germline-encoded antibody that possesses broad crossreactivity to the glycine-alanine region. Because these antibodies require few if any mutations, they should have high precursor frequencies and should be very similar in all individuals. Experiments to test these ideas are presently in progress.

The autoantibodies we describe also fulfill the expectations of the epitope homology (mimicry) model. They appear in all patients who produce antibodies to the viral protein during acute infection, they form a crossreactive set of proteins, and they are inhibited by a common sequence derived from the viral protein. These data, taken together, form a strong case for molecular mimicry in generating autoantibody production during IM, and they offer the first demonstration of the specific structure within a molecule responsible for the homology.

### Summary

Viruses have been postulated to be involved in the induction of autoantibodies by: (a) autoimmunization with tissue proteins released by virally induced tissue damage; (b) immunization with virally encoded antigens bearing molecular

similarities to normal tissue proteins; or (c) nonspecific (polyclonal) B cell stimulation by the infection. Infectious mononucleosis (IM) is an experiment of nature that provides the opportunity for examining these possibilities. We show here that IgM antibodies produced in this disease react with at least nine normal tissue proteins, in addition to the virally encoded Epstein-Barr nuclear antigen (EBNA-1). The antibodies are generated to configurations in the glycine-alanine repeat region of EBNA-1 and are crossreactive with the normal tissue proteins through similar configurations, as demonstrated by the effectiveness of a synthetic glycine-alanine peptide in inhibiting the reactions. The antibodies are absent in preillness sera and gradually disappear over a period of months after illness, being replaced by IgG anti-EBNA-1 antibodies that do not crossreact with the normal tissue proteins but that are still inhibited by the glycine-alanine peptide. These findings are most easily explained by either a molecular mimicry model of IgM autoantibody production or by the polyclonal activation of a germline gene for a crossreactive antibody. It also indicates a selection of highly specific, non-crossreactive anti-EBNA-1 antibodies during IgM to IgG isotype switching.

We thank Drs. Richard Smith, Charles Horowitz, Sherman Fong, Sallie Hoch, and Georg Reimer for stimulating discussion and providing materials and advice.

*Received for publication 1 December 1986.*

### References

1. Sutton, R. N. P., R. T. D. Emond, D. B. Thomas, and D. Doniach. 1974. The occurrence of autoantibodies in infectious mononucleosis. *Clin. Exp. Immunol.* 17:427.
2. Mead, G. M., P. Cowin, and J. M. A. Whitehouse. 1980. Antitubulin antibody in healthy adults and patients with infectious mononucleosis and its relationship to smooth muscle antibody (SMA). *Clin. Exp. Immunol.* 39:328.
3. Linder, E., P. Kurki, and L. C. Andersson. 1979. Autoantibody to "intermediate filaments" in infectious mononucleosis. *Clin. Immunol. Immunopathol.* 14:411.
4. Kataaha, P. K., S. M. Mortazavi-Milani, G. Russell, and E. J. Holborow. 1985. Anti-intermediate filament antibodies, antikeratin antibody, and antiperinuclear factor in rheumatoid arthritis and infectious mononucleosis. *Ann. Rheum. Dis.* 44:446.
5. Gripenberg, M., and E. Linder. 1984. Demonstration of anti-keratin antibodies by ELISA using keratin or thiol-containing compounds in urea as antigens. *J. Immunol. Methods.* 75:65.
6. Toh, B. H., A. Yildiz, J. Sotelo, O. Osung, E. J. Holborow, F. Kanakoudi, and J. V. Small. 1979. Viral infections and IgM autoantibodies to cytoplasmic intermediate filaments. *Clin. Exp. Immunol.* 37:76.
7. Kurki, P., and I. Virtanen. 1984. The detection of human antibodies against cytoskeletal components. *J. Immunol. Methods* 67:209.
8. Haire, M. 1972. Fibrillar anti-cellular antibody associated with mumps and measles infection. *Clin. Exp. Immunol.* 12:335.
9. Kaplan, M. H., and J. D. Frengley. 1969. Autoimmunity to the heart in cardiac disease: Current concepts of the relation of autoimmunity to rheumatic fever, post-cardiotomy and post-infarction syndromes and cardiomyopathies. *Am. J. Cardiol.* 24:459.
10. Van de Rijn, I., J. B. Zabriskie, and M. McCarty. 1977. Group A streptococcal antigens cross-reactive with myocardium. *J. Exp. Med.* 146:579.

11. Haspel, M. V., T. Onodera, B. S. Prabhakar, M. Horitz, H. Suzuki, and A. L. Notkins. 1983. Virus-induced autoimmunity: Monoclonal antibodies that react with endocrine tissues. *Science (Wash. DC)*. 220:304.
12. Fagraeus, A., C. Orvell, R. Norberg, and E. Norrby. 1983. Monoclonal antibodies to epitopes shared by actin and vimentin obtained by paramyxovirus immunization. *Exp. Cell Res.* 145:425.
13. Fujinami, R. S., M. B. A. Oldstone, Z. Wroblewska, M. E. Frankel, and H. Koprowski. 1983. Molecular mimicry in virus infection: Crossreaction of measles virus phosphoprotein or of herpes simplex virus protein with human intermediate filaments. *Proc. Natl. Acad. Sci. USA*. 80:2346.
14. Dales, S., R. S. Fujinami, and M. B. A. Oldstone. 1983. Infection with vaccinia favors the selection of hybridomas synthesizing autoantibodies against intermediate filaments, one of them cross-reacting with the virus hemagglutinin. *J. Immunol.* 131:1546.
15. Srinivasappa, J., J. Saegusa, B. S. Prabhakar, M. K. Gentry, M. J. Buchmeier, T. J. Wiktor, H. Koprowski, M. B. A. Oldstone, and A. L. Notkins. 1986. Molecular mimicry: frequency of reactivity of monoclonal antiviral antibodies with normal tissues. *J. Virol.* 57:397.
16. Wise, K. S., and R. K. Watson. 1985. Antigenic mimicry of mammalian intermediate filaments by mycoplasmas. *Infect. Immun.* 48:587.
17. Slaughter, L., D. A. Carson, F. C. Jensen, T. L. Holbrook, and J. H. Vaughan. 1978. In vitro effects of Epstein-Barr virus on peripheral blood mononuclear cells from patients with rheumatoid arthritis and normal subjects. *J. Exp. Med.* 148:1429.
18. Fong, S., J. H. Vaughan, C. D. Tsoukas, and D. A. Carson. 1982. Selective induction of autoantibody secretion in human bone marrow by Epstein-Barr virus. *J. Immunol.* 129:1941.
19. Robinson, J. E., and K. C. Stevens. 1984. Production of autoantibodies to cellular antigens by human B cells transformed by Epstein-Barr virus. *Clin. Immunol. Immunopathol.* 33:339.
20. Garzelli, C., F. E. Taub, J. E. Scharff, B. S. Prabhakar, F. Ginsberg-Fellner and A. L. Notkins. 1984. Epstein-Barr virus-transformed lymphocytes produce monoclonal autoantibodies that react with antigens in multiple organs. *J. Virol.* 52:722.
21. Henle, W., and G. Henle. 1982. Immunology of Epstein-Barr virus. *In The Herpesviruses*, Vol. 1. Plenum Press, New York. pg. 204.
22. de Thé, G. 1982. Epidemiology of Epstein-Barr virus and associated disease in man. *In The Herpesviruses*, Vol. 1. Plenum Press, New York. pg. 25.
23. Yates, J. L., N. Warren, and B. Sugden. 1985. Stable replication of plasmids derived from Epstein-Barr virus in various mammalian cells. *Nature (Lond.)*. 313:812.
24. Alspaugh, M. A., F. C. Jensen, H. Rabin, and E. M. Tan. 1978. Lymphocytes transformed by the Epstein-Barr virus. Induction of nuclear antigen reactive with antibody in rheumatoid arthritis. *J. Exp. Med.* 147:1018.
25. Billings, P. B., S. O. Hoch, P. J. White, D. A. Carson, and J. H. Vaughan. 1983. Antibodies to the Epstein-Barr virus nuclear antigen and to rheumatoid arthritis nuclear antigen identify the same polypeptide. *Proc. Natl. Acad. Sci. USA*. 80:7104.
26. Rhodes, G., D. A. Carson, J. Valbracht, R. Houghten, and J. H. Vaughan. 1985. Human immune responses to synthetic peptides from the Epstein-Barr nuclear antigen. *J. Immunol.* 134:211.
27. Hennessy, K., M. Heller, V. van Santen, and E. Kieff. 1983. Simple repeat array in Epstein-Barr virus DNA encodes part of the Epstein-Barr nuclear antigen. *Science (Wash. DC)*. 220:1396.

28. Hennessy, K., and E. Kieff. 1983. One of two Epstein-Barr virus nuclear antigens contains a glycine-alanine copolymer domain. *Proc. Natl. Acad. Sci. USA.* 80:5665.
29. Baer, R., A. T. Bankier, M. D. Biggin, P. L. Deininger, P. J. Farrell, T. J. Gibson, G. Hatfull, G. S. Hudson, S. C. Satchwell, C. Seguin, P. S. Tuffnell, and B. G. Barrell. 1984. DNA sequence and expression of the B95-8 Epstein-Barr virus genome. *Nature (Lond.)*. 310:207.
30. Heller, M., A. Henderson, and E. Kieff. 1982. A repeat sequence in Epstein-Barr virus DNA is related to interspersed repeated cell DNAs which are at specific sites on human chromosomes. *Proc. Natl. Acad. Sci. USA.* 79:5916.
31. Arrand, J., J. Walsh-Arrand, and L. Rymo. 1983. Cytoplasmic RNA from normal and malignant human cells shows homology to the DNAs of Epstein Barr virus and human adenovirus. *EMBO (Eur. Mol. Biol. Organ.) J.* 2:1673.
32. Heller, M., E. Flemington, E. Kieff, and P. Deininger. 1985. Repeat arrays in cellular DNA related to EBV IR3 repeat. *Mol. Cell. Biol.* 5:457.
33. Seibl, R., and H. Wolf. 1985. Mapping of Epstein-Barr virus proteins on the genome by translation of hybrid-selected RNA from induced P3HR1 cells and induced Raji cells. *Virology.* 141:1.
34. Luka, J., T. Kreofsky, G. R. Pearson, K. Hennessy, and E. Kieff. 1984. Identification and characterization of a cellular protein that cross-reacts with the Epstein-Barr virus nuclear antigen. *J. Virol.* 52:833.
35. Epstein, A. L., R. Levy, H. Kim, W. Henle, G. Henle, and H. S. Kaplan. 1978. Biology of the human malignant lymphomas. IV. Functional characterization of ten diffuse histiocytic lymphoma cell lines. *Cancer (Phila.)*. 42:2379.
36. Winter, J. N., D. Variakojis, and A. L. Epstein. 1984. Phenotypic analysis of established diffuse histiocytic lymphoma cell lines utilizing monoclonal antibodies and cytochemical techniques. *Blood.* 63:140.
37. Peterson, G. L. 1977. A simplification of the protein assay method of Lowry et al. which is more generally applicable. *Anal. Biochem.* 83:346.
38. Peterson, G. L. 1978. Review of the folin phenol protein quantitation method of Lowry, Rosebrough, Farr and Randall. *Anal. Biochem.* 100:201.
39. Peterson, G. L. 1983. Determination of total protein. *Methods Enzymol.* 91:95.
40. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265.
41. Billings, P. B., and S. O. Hoch. 1983. Isolation of intact Sm/RNP antigens from rabbit thymus. *J. Immunol.* 131:347.
42. Rhodes, G., R. A. Houghten, D. A. Carson, J. Valbracht, and J. H. Vaughan. 1984. Mapping the antigenic regions of Epstein-Barr nuclear antigen using synthetic peptides. In *Herpesvirus*. F. Rapp, editor. Alan R. Liss, New York. pg. 487.
43. Merrifield, R. B. 1963. Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* 85:2149.
44. Houghten, R. A., W. C. Chang, and C. H. Li. 1980. Human beta-endorphin: synthesis and characterization of analogs iodinated and tritiated at tyrosine residues 1 and 27. *Int. J. Pept. Protein Res.* 16:311.
45. Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA.* 76:4350.
46. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of protein for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* 29:185.
47. Barka, T., and P. J. Anderson. 1963. Histochemistry. Chapter X. Harper and Row, New York. pg. 227.
48. Smith, R. S., G. Rhodes, J. H. Vaughan, C. A. Horwitz, J. E. Geltosky, and A. S.

- Whalley. 1986. A synthetic peptide for detecting antibodies to Epstein-Barr virus nuclear antigen in sera from patients with infectious mononucleosis. *J. Infect. Dis.* 154:885.
49. Strnad, B. C., T. C. Schuster, R. F. Hopkins, R. H. Neubauer, and H. Rabin. 1981. Identification of an Epstein-Barr virus nuclear antigen by fluoroimmuno-electrophoresis and radioimmuno-electrophoresis. *J. Virol.* 38:990.
50. Ochs, D. 1983. Protein contaminants of sodium dodecyl sulfate-polyacrylamide gels. *Anal. Biochem.* 135:470.
51. Rumpold, H., G. H. Rhodes, P. L. Bloch, D. A. Carson, and J. H. Vaughan. 1987. The glycine-alanine repeating region is the major epitope of the Epstein-Barr nuclear antigen-1 (EBNA-1). *J. Immunol.* 138:593.
52. Hanukogiu, I., and E. Fuchs. 1982. The cDNA sequence of a human epidermal keratin: Divergence of sequence but conservation of structure among intermediate filament proteins. *Cell.* 31:243.
53. Johnson, L. B., W. W. Idler, X.-M. Zhou, D. R. Roop, and P. M. Steinert. 1985. Structure of a gene for the human epidermal 67-kDa keratin. *Proc. Natl. Acad. Sci. USA.* 82:1896.
54. Rhodes, G., R. Houghten, J. P. Taulane, D. A. Carson, and J. H. Vaughan. 1984. The immune response to Epstein-Barr nuclear antigen: Conformational and structural features of antibody to synthetic peptides. *Mol. Immunol.* 21:1047.
55. Scott, D. W. 1985. Mechanisms in immune tolerance. *CRC Crit. Rev. Immunol.* 5:1.