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# Polypeptides of the Epstein-Barr Virus Membrane Antigen Complex

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Epstein-Barr virus (EBV)-associated membrane antigens have been purified from the plasma membranes of the producer cell line P3HR-1 NONO. The antigens were assayed with a specific rabbit anti-EBV antiserum using an $^{125}$I-labeled staphyloccocal protein A binding assay. The antigens have been shown to be present on purified plasma membranes. Treatment of the plasma membranes with Triton X-100 allows the separation of two antigenically distinct classes of antigens, one soluble and one insoluble in the detergent. Immunoprecipitates of [$^{125}$I]- and [$^{35}$S]methionine-labeled, detergent-soluble antigens contained three major polypeptides of molecular weights of 350,000, 140,000, and 75,000 (on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and several minor components. These polypeptides were all specifically precipitated from four EBV-producer cell lines, P3HR-1, P3HR-1 NONO, B95-8, and 7744. They could not be precipitated from producer cell lines treated with phosphonoacetic acid, which inhibits late viral functions, nor could they be precipitated from nonproducer cell lines. The 350,000 and 75,000 molecular weight polypeptides bound to Ricin and lentil lectin columns; however, most of the 140,000 molecular weight material did not. A component of molecular weight 220,000 (prominent only in P3HR-1 NONO) was probably a degradation product of the 350,000 molecular weight polypeptide.

Epstein-Barr virus (EBV) is a large, complex virus 11 nm in diameter (8) with a genome of $10^8$ daltons (32). The virus is able to transform normal human peripheral B lymphocytes in vitro into permanently growing lymphoblastoid cell lines (15) which express the Epstein-Barr nuclear antigen (25) and carry multiple copies of the viral genome (33). Virtually nothing is known about the mechanism of this process or the nature and function of the Epstein-Barr nuclear antigen, although the latter has recently been studied intensively (1, 8, 19).

Lytic infection by EBV has been demonstrated in vivo in humans (20) and in vitro with new world primates (21). EBV infection is associated, in some circumstances, with certain disease states, i.e., infectious mononucleosis (14), African Burkitt's lymphoma (12) and nasopharyngeal carcinoma (15). Again, very little is known about the process whereby virus is released or about the structure of the virus itself at the molecular level.

In a previous study (28), a rabbit anti-EBV antiserum of high neutralizing titer was produced. The neutralizing antibody could be specifically absorbed only by EBV producer cells, indicating that EBV producer cells express antigens on their surfaces which cross-react with the neutralizing antigens on the virus. Furthermore, these antigens are only expressed on the small percentage of cells which are actively producing virus. The isolation of neutralizing antigens is of interest for two reasons: first, it is thought that antibodies against these antigens can protect against reinfection by the virus and therefore, purified or partially purified antigen preparations may be useful in vaccination studies (5, 7); second, they provide information about the structural polypeptides of the virus and their assembly into an envelope during virus maturation. In this paper, we report the purification and characterization of the EBV membrane antigen complex from the plasma membranes of EBV producer cell lines.

## MATERIALS AND METHODS

**Cells.** The method of maintenance of the cell lines used in this study has been described elsewhere (28). Large-scale cultures of P3HR-1 NONO were carried in diptheria toxin bottles (VRW). The bottles, containing 2 liters of culture, were stood on end overnight before harvesting. The next day the bulk of the supernatant was siphoned off before removal of the cells for centrifugation. This procedure reduced the volume to

be centrifuged to obtain the cell pellet by a factor of 10.

The preparation of the immune and preimmune rabbit anti-EBV sera has been described previously (28). The rabbit anti-human T cell serum A99 and the rabbit anti-$\beta_2$-microglobulin were kind gifts from Diane Pratt.

**$^{125}$I-labeled staphyloccal protein A competition radioimmunoassay.** Antigen preparations were assayed after normalization of the Triton X-100 (Sigma Chemical Co.) concentration to 0.1%. The antigen (50 μl) was incubated overnight with antibody (10 μl of 1:8 diluted rabbit anti-EBV serum) at 4°C. The antibody activity in duplicate 25-μl portions was then assayed as described previously (28), except that the P3HR-1 NONO cells which were generally used in the assay were fixed with glutaraldehyde immediately before the assay. The cells were washed once in Hanks balanced salt solution, supplemented with 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.2), and resuspended to $1 \times 10^6$/ml in the same buffer, and an equal volume of 0.25% glutaraldehyde (Sigma) in the same buffer was added. After 5 min at 20°C, the reaction was stopped by addition of bovine serum albumin (Miles Laboratories, Inc.) in the same buffer and the cells were washed twice before use.

The antigenic activity of a preparation in a total volume ($v$ μl) was defined in units as being equal to the fold dilution of 50 μl of antigen which would absorb 50% of the binding activity of 10 μl of 1:8 diluted antibody $\times$ $v/50$.

Since the Triton X-100-soluble antigens competitively inhibited the assay to a plateau of 60 to 70% (see Results), the 50% inhibition point was always calculated from this curve, i.e., with a range of 0–65% the 50% point would be taken as 32.5% inhibition. This was then used as a reference for measurement on further purified material. A new calibration curve using Triton X-100-solubilized material was made for every assay. All data were calculated after subtracting the background of binding shown by a negative preimmune serum. This background was usually about 30% of that obtained with positive sera.

**Purification of plasma membranes.** Purification of plasma membranes was based on the method of Crumpton and Snary (4). All procedures were carried out at 4°C. Cells were obtained by centrifugation at $250 \times g$ for 10 min in the JA 10 Beckman rotor in the J216 Beckman preparative centrifuge. Usually 10 to 15 ml of packed cells was resuspended to 100 ml in RPMI 1640 and subjected to nitrogen cavitation with a Parr model 4635 cell disruption bomb at a pressure of 250 lb/in$^2$ for 5 to 10 min. The crude nuclei were then removed by centrifugation at $600 \times g$ for 10 min in a Sorvall RC3 centrifuge. The nuclei were washed once, and the total supernatant was centrifuged at $4{,}000 \times g$ for 15 min in the RC5 centrifuge. The supernatant was poured off from the crude mitochondrial pellet, and the crude membranes were pelleted by centrifugation at $50{,}000 \times g$ for 1 h in the SS34 rotor of an RC-5 centrifuge. The resulting pellets were resuspended by using a tight-fitting Dounce homogenizer in 10 mM Tris (pH 7.4). The suspension was then adjusted to 40% sucrose (Schwarz/Mann density grade) by addition of 60% sucrose and divided equally among three cellulose nitrate centrifuge tubes (Beckman Instruments Inc.; 1 by 3.5 in. [2.54 by 8.8 cm]). The tubes were half-filled with 40% sucrose in 10 mM Tris (pH 7.4) and overlaid with 27% sucrose in 10 mM Tris (pH 7.4). The sucrose gradients were centrifuged overnight at 23,000 rpm ($g$ average, 75,000) in an SW27 swinging bucket rotor of an L5-65 ultracentrifuge. The interface plasma membrane material was removed, diluted 1:3 (vol/vol) with 50 mM Tris (pH 7.4), and pelleted for 1 h at $50{,}000 \times g$ in the Sorvall RC-5 as before. The pellets were resuspended as before. The amount of protein and DNA in the preparation was estimated by the method of Warburg and Christian (31), by measuring the absorbancy at 280 nm ($A_{280}$) and $A_{260}$ of a sample after boiling in sodium dodecyl sulfate (SDS). By using their correction to estimate the true $A_{280}$ and assuming that a 1-mg/ml protein solution has an $A_{280}$ of 1, then the typical yield of plasma membrane protein was approximately 2 mg/ml of packed cells, with a DNA content of 4 to 6% by weight. The plasma membranes were solubilized by the addition of detergent, usually Triton X-100, in a ratio of 1 mg of detergent per mg of protein based on the preceding measurement. The mixture was then made up to 5 ml with 10 mM Tris (pH 7.4) and incubated at 4°C for 30 min. After this time, the mixture was centrifuged at 50,000 rpm ($g$ average, ≃200,000) in an SW50.1 swinging bucket rotor of a Beckman L5-65 ultracentrifuge.

**Removal of excess detergent.** For some experiments detergent levels were lowered before assay by mixing with polystyrene beads (SM-2 Bio-Rad). The details of this procedure have been published previously (29).

**Lectin affinity chromatography.** The following Sepharose-coupled lectins were obtained from Vector Labs: *Lens culinaris* agglutinin (lentil), *Ricinus communis* agglutinin II (Ricin), *Ulex europaeus* agglutinin I, and wheat germ agglutinin. Sepharose-coupled *Helix pomatia* lectin was the kind gift of Diane Pratt.

Samples were loaded onto the affinity columns (2-ml columns for large-scale preparations, 0.5-ml columns for small-scale [$^{35}$S]methionine-labeled preparations) at the maximum flow rate. All columns were preequilibrated with a solution of 0.2% Triton X-100, 0.1 mM $MnCl_2$, 0.14 M NaCl, 0.25 mM dithiothreitol, and 10 mM Tris, pH 7.8 (lectin buffer) and washed with 5- to 10-column volumes of Lectin buffer after application of the sample. The appropriate sugar in 1 ml of lectin buffer was applied to the column, which was then turned off and allowed to stand for 30 min. The specifically bound material was then eluted by using the same sugar solution. All procedures were performed at 4°C. Sugars were purchased from Sigma and used at 3% (wt/vol) in lectin buffer, except for galactose which was 10% (wt/vol).

**Lactoperoxidase- and chloramine T-catalyzed iodination.** These iodination procedures were performed exactly as described in the literature (3, 11).

**Metabolic labeling with [$^{35}$S]methionine.** Cells ($2 \times 10^7$) were pelleted at 1,000 rpm in an RC-3 centrifuge and resuspended in 15 ml of methionine-

free RPMI 1640 (ABS) supplemented with 5% dialyzed fetal calf serum, and 0.5 mCi of [$^{35}$S]methionine (800 Ci/mmol) (Amersham Corp.). The cells were incubated at 37°C overnight, pelleted, resuspended in 0.1 ml of saline, and lysed by the addition of 2 ml of a solution of 0.5% Triton X-100, 1 mM $MgCl_2$, 1 mM $\alpha$-toluenesulphonyl fluoride, 0.25 mM dithiothreitol, and 10 mM Tris (pH 7.4). The mixture was incubated at 4°C for 0.5 h and then centrifuged at 50,000 rpm (*g* average, 150,000) in a type 50 Ti rotor in an L5-65 Beckman ultracentrifuge that uses 10-ml Oak Ridge polycarbonate tubes. The resulting supernatant was then passed over a Ricin lectin column as described above.

**Immunoprecipitation with fixed *Staphylococcus aureus*.** Immunoprecipitation was exactly as described in the literature (17), except for the B95-8 and B95-8 PAA cell lines. In these cases a preclearing step was required because these lines possess two major polypeptides which bind very strongly to *S. aureus*. The material ($1 \times 10^7$ to $2 \times 10^7$ cpm in 100 to 200 $\mu$l) was precleared by addition of 5 to 10 $\mu$l of preimmune rabbit serum, followed by incubation at 4°C for 1 h. The suspension of *S. aureus* was then added (10 $\mu$l of 10% suspension per 1 $\mu$l of serum) and incubated for an additional 1 h. The suspension was then clarified. The immunoprecipitations in any given experiment were performed on equal amounts of material from each cell line as judged by the amount of isotopic label in the Ricin eluate.

**SDS-PAGE.** The SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (28). Myosin (Bio-Rad Laboratories), thyroglobulin (Sigma), and LETS protein from Lan Bo Chen were included as molecular weight markers.

**Protein estimation.** In early experiments with purified plasma membranes the protein concentration was estimated from the ratio of $A_{280}$ to $A_{260}$ (see plasma membrane preparation above). However, Triton X-100 absorbs strongly in this range; therefore, protein estimates in later experiments were performed by the Lowry method (bovine serum albumin standard) with the modifications of Bennett (2) and Dulley and Grieve (6). For whole plasma membrane protein the Lowry procedure gave values approximately 30% lower than for the absorbance method. The method used for protein determination is indicated in the appropriate table and figure legends.

## RESULTS

**Demonstration of the EBV-associated membrane antigen on purified plasma membranes and its solubilization by Triton X-100.** It was demonstrated previously that a rabbit serum prepared against whole purified EBV (rabbit anti-EBV) could be used to detect quantitatively the EBV-membrane antigen complex on the surface of producer cell lines by means of the $^{125}$I-labeled *S. aureus* protein A assay (23, 28). By competing for the antibody before the assay, it is possible to quantitate the amount of antigen in a given preparation. A competition curve using P3HR-1 NONO cells as the source of competing antigen is presented in Fig. 1. P3HR-1 NONO was chosen as the cell line for both assay and purification studies because it expressed the most membrane antigen per cell when grown under stressed conditions (see Materials and Methods) (28). Furthermore, the rabbit antiserum had been raised against the B95-8 strain of EBV, which was derived from a marmoset cell line; therefore, use of the human P3HR-1 NONO cell line in purification experiments would minimize the possibility of studying spurious reactivities in the serum.

Plasma membranes were prepared from P3HR-1 NONO cells by nitrogen cavitation and discontinuous density centrifugation. The ability of such a membrane preparation to inhibit the $^{125}$I-labeled protein A binding assay is shown in Fig. 2. This experiment indicates that equal amounts of activity were present in the purified plasma membrane faction and in the endoplasmic reticulum. This was not due to the presence of contaminating plasma membranes in the endoplasmic reticulum, since less than 10% of the $\beta_2$-microglobulin was associated with the endoplasmic reticulum fraction.

Ultracentrifugation of the plasma membrane preparation (100,000 × *g* per h) removes the EBV membrane antigen activity from the supernatant (Fig. 3). If, however, the plasma membranes were ultracentrifuged after preincubation with Triton X-100 (1:1 [wt/wt], Triton to protein for 30 min at 4°C), activity remained in the supernatant, indicating that the antigen complex

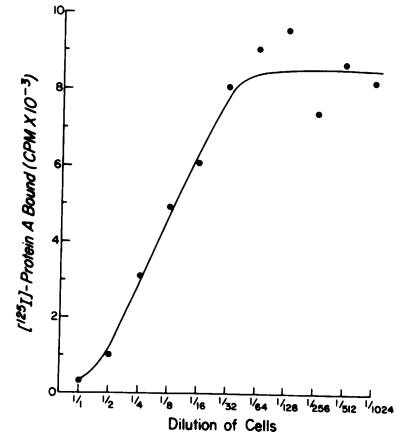


FIG. 1. *Absorption of the rabbit anti-EBV antiserum by membrane antigens on P3HR-1 NONO cells. Serially diluted cells ($10^7$/ml, 100 $\mu$l) were placed in V-shaped microtiter plates and pelleted. Anti-EBV antiserum (100 $\mu$l) was added at a dilution of 1:50, and the cells were resuspended. The suspensions were rocked overnight at 4°C. The cells were pelleted, and the activity of the antiserum was assayed by the $^{125}$I-labeled protein A binding assay (28).*

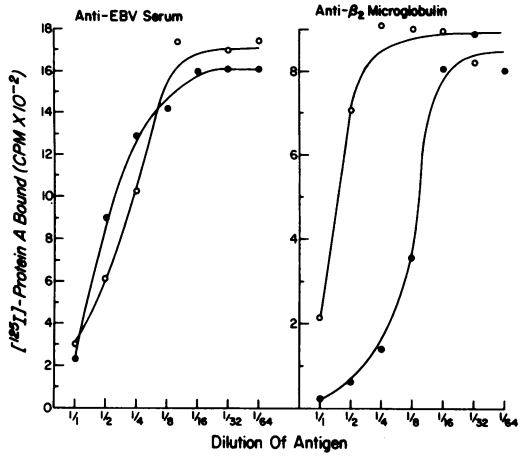


FIG. 2. *Detection of EBV-membrane antigens and $\beta_2$-microglobulin on purified plasma membranes and crude endoplasmic reticulum. Membranes from the P3HR-1 NONO line were assayed as described in Materials and Methods. An antigen dilution of 1 is equivalent to 186 μg of membrane protein estimated by $A_{280}$. The plasma membranes were removed from the antiserum by centrifugation before assay. Symbols: ○, crude endoplasmic reticulum; ●, purified plasma membrane.*

was solubilized under these conditions. A characteristic feature of this kind of experiment was the failure of the Triton X-100-solubilized material to compete completely in the assay. No matter how much of the solubilized material was added, it was never possible to obtain more than 60 to 70% inhibition (Fig. 3). In contrast, the antigens in the total plasma membrane after Triton X-100 treatment, but before centrifugation, inhibited by almost 100%. These results suggest that the antiserum contains antibodies against two distinct antigenic systems in the membrane antigen complex, one soluble and one insoluble in Triton X-100.

By using Triton X-100, 60 to 70% of the soluble antigenic activity was extracted compared with only 35 to 45% of the membrane protein (Table 1). By comparison, the nonionic detergent Brij-94 only solubilized 30 to 40% of the activity, and the ionic detergent sodium deoxycholate interfered with the assay for reasons which are not known. Therefore, Triton X-100 was used in all subsequent studies.

When the Triton X-100-solubilized material was used to compete for the binding of the T cell specific antiserum A99 to the T cell line CEM, no inhibition was seen (Fig. 4). Furthermore, mock-purified material from the EBV-negative cell line RAMOS did not compete for the binding of the rabbit anti-EBV serum to P3HR-1 NONO cells (Fig. 5). These two experiments demonstrated that the solubilized plasma membrane preparation from P3HR-1 NONO was specifically competing for the anti-EBV serum in the $^{125}$I-labeled protein A assay. Therefore, the purified plasma membranes contain at least part of the EBV-membrane antigen complex.

**Purification of the EBV-membrane antigen complex by lectin affinity chromatography.** One of the most effective methods for purifying membrane proteins is based on the fact that many of them are glycosylated and will bind specifically to lectin affinity columns. In Table 2, data are summarized from one experiment in which the ability of several lectins to bind the EBV-membrane antigen complex was studied. The membrane antigens bound to Ricin affinity columns, resulting in approximately 50-fold purification in high yield after elution with galactose. The antigens also bound partially to lentil lectin (60% of the activity was specifically bound, the rest passed through), suggesting heterogeneity in the glycosylation of the membrane

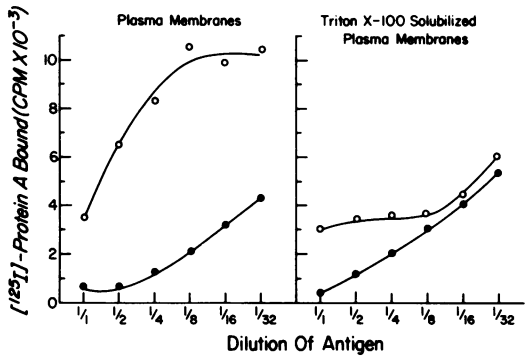


FIG. 3. *Solubilization of EBV membrane antigens by Triton X-100. Plasma membranes from P3HR-1 NONO (antigen dilution of 1 = 3.3 mg of membrane protein by $A_{280}$) were preincubated with either Triton X-100 (1 mg/mg of protein) or 10 mM Tris (pH 7.4) for 0.5 h in a final volume of 300 μl. Half of each sample was then subjected to ultracentrifugation at 100,000 × g for 1 h. The samples were then assayed as described in the legend to Fig. 1. Symbols: ●, fraction before 100,000 × g for 1 h of treatment; ○, supernatant after 100,000 × g for 1 h of treatment.*

TABLE 1. *Recovery of EBV-membrane antigens and total protein after solubilization of P3HR-1 NONO membranes in Triton X-100*

| Membrane | Activity (U/ml) | Lowry protein (mg/ml) | Sp act (U/mg) | Recovery (%) | |
|---|---|---|---|---|---|
| | | | | Activity | Protein |
| Plasma | 1,120 | 6.9 | 162 | | |
| Triton-solubilized | 666 | 2.6 | 256 | 59 | 38 |

antigen. Furthermore, recovery of total activity from the lentil lectin columns was variable and often low (<30%). Therefore, this method was not effective as a purification step. No antigenic difference between bound and unbound material from the lentil column was detected by the $^{125}$I-labeled protein A assay. The antigens did not bind to wheat germ agglutinin (Table 2), nor did they bind to the lectin *U. europaeus* agglutinin I or to the lectin isolated from *H. pomatia* (data not shown).

Inhibition curves on equivalent samples of the Triton X-100-solubilized plasma membrane material and on the material purified by Ricin affinity chromatography (Fig. 6) were identical, indicating that most of the membrane antigen specificities present in the soluble plasma membrane preparation were recovered from the affinity column. Furthermore, material from the EBV-negative cell line RAMOS after mock purification over the Ricin column had no inhibitory activity (data not shown).

**Immunoprecipitation of the membrane antigens from material purified by passage over a ricin affinity column.** Immunoprecipitation of membrane proteins purified by Ricin affinity chromatography was used as a final purification step to identify the components of the membrane antigen complex. The material was labeled in two ways: first by chloramine T-catalyzed iodination with $^{125}$I; and second by metabolic incorporation of [$^{35}$S]methionine. The SDS-PAGE pattern of the immunoprecipitate obtained from P3HR-1 NONO after labeling with $^{125}$I is presented in Fig. 7. Two major bands were seen with molecular weights of approximately 220,000 and 75,000. The SDS-PAGE pattern of the immunoprecipitate from [$^{35}$S]methionine-labeled B95-8 is shown in Fig. 8. Included in this experiment were B95-8 cells grown in the presence of phosphonoacetic acid, which inhibits expression of late viral functions in producer cell lines (10, 22, 28). Two major bands were precipitated from B95-8, but had molecular weights of 350,000 and 75,000 and, in addition, several minor bands including those with molecular weights of 220,000, 140,000, and 56,000. The same result was obtained when immunoprecipitation was performed on material purified by

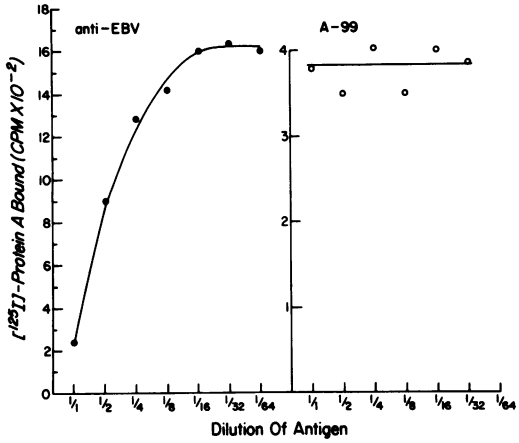


FIG. 4. *Absorption of the anti-EBV serum and an anti-human T cell serum A99 by P3HR-1 NONO plasma membranes. For details see the legend to Fig. 2.*

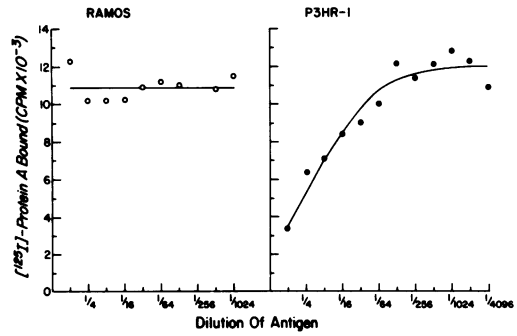


FIG. 5. *Absorption of the anti-EBV serum by Triton X-100-solubilized P3HR-1 NONO and RAMOS plasma membranes. In this experiment an antigen dilution of 1 is equivalent to 60 μg of Triton X-100-soluble protein (assay by Lowry). The antigen was assayed as described in Materials and Methods.*

TABLE 2. *Binding of Triton X-100-solubilized EBV membrane antigens to lectin affinity columns*

| Agglutinin[a] | Activity (U) 840[b] | | Lowry protein (μg) 720[b] | | Recovery (%) | | Sp act (U/mg) 1,200[b] | |
|---|---|---|---|---|---|---|---|---|
| | Flow through | Eluate | Flow through | Eluate | Activity | Protein | Flow through | Eluate |
| WGA | 240 | 230 | 430 | 120 | 56 | 76 | 560 | 1,900 |
| Lentil | 160 | 230 | 570 | 7[c] | 46 | 80 | 280 | 33,000[c] |
| Ricin | 100 | 730 | 410 | 10[c] | 99 | 58 | 240 | 73,000[c] |

[a] WGA, Wheat germ agglutinin; lentil, *Lens culinaris* agglutinin; Ricin, *Ricinus communis* agglutinin II.
[b] Numbers indicate activity, Lowry protein, and specific activity in the load.
[c] The protein estimates here are at the limit of sensitivity of the Lowry assay and can be taken only as an indication of protein concentration.

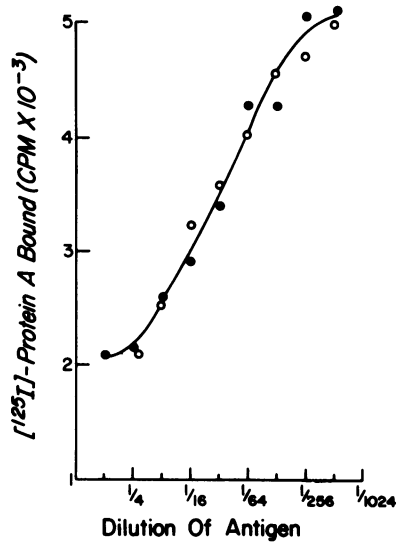


FIG. 6. *Absorption of the anti-EBV serum by Triton X-100-solubilized plasma membranes before and after passage over a Ricin lectin affinity column. In this experiment an antigen dilution of 1 is equivalent to 85 μg of Triton X-100-soluble protein (assay by Lowry). The antigen eluted from the Ricin column was adjusted to the same volume as the applied sample before assay. Symbols: ●, before passage over a Ricin lectin affinity column; ○, after passage over a Ricin lectin affinity column.*

lentil lectin affinity chromatography. The pattern obtained with B95-8 was the cleanest of all the producer lines studied and was reproducible for both major and minor bands. The molecular weight of the largest band was estimated from 5% polyacrylamide gels, assuming a linear extrapolation from the molecular weight markers myosin (200,000) and LETS protein (220,000). The polypeptide also migrated closely to the heaviest subunit of thyroglobulin (330,000) (30). The 75,000 molecular weight polypeptide electrophoresed anomalously on different percentage gels, migrating with an apparent weight of 60,000 on 5% gels and 75,000 on 7.5% gels. This is presumably due to the presence of glycan chains on the protein. No bands were seen when immunoprecipitation was performed with preimmune sera or with immune sera on B95-8 cells treated with phosphonoacetic acid.

Immunoprecipitation was performed on [$^{35}$S]methionine-labeled P3HR-1 cells grown under stressed (P3HR-1 NONO) or normal conditions with or without phosphonoacetic acid (Fig. 9). As seen before, the P3HR-1 NONO gave a major polypeptide of 220,000 compared with 350,000 from B95-8. Growth of the P3HR-1 under normal conditions gave bands of both mo-

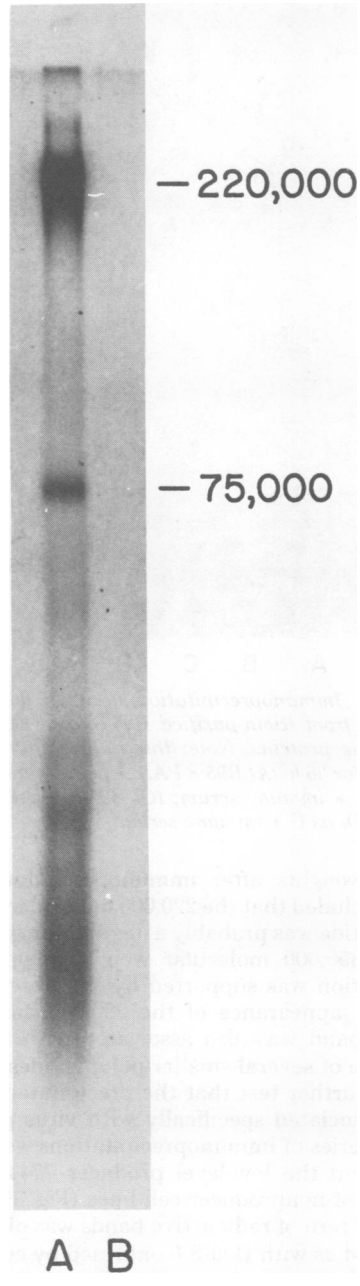


FIG. 7. *Immunoprecipitation of EBV membrane antigens by anti-EBV serum from material purified by Ricin lectin affinity chromatography. EBV membrane antigens from the P3HR-1 NONO cell line solubilized in Triton X-100 and purified over Ricin lectin affinity columns were iodinated with $^{125}I$ by the chloramine T method (see Materials and Methods) and then subjected to immunoprecipitation. (A) Immune rabbit anti-EBV serum; (B) preimmune serum.*

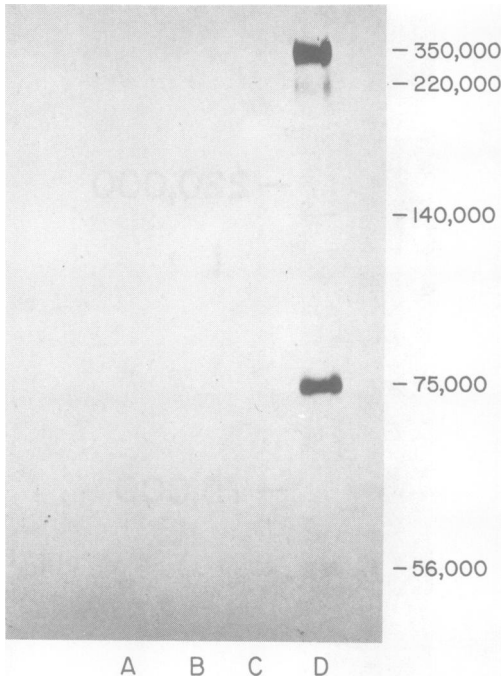


FIG. 8. *Immunoprecipitation of EBV membrane antigens from Ricin-purified B95-8 and B95-8 PAA membrane proteins. Note: this autoradiogram was exposed for 36 h. (A) B95-8 PAA + preimmune serum; (B) as A + immune serum; (C) B95-8 + preimmune serum; (D) as C + immune serum.*

lecular weights after immunoprecipitation. It was concluded that the 220,000 molecular weight polypeptide was probably a degradation product of the 350,000 molecular weight polypeptide. This notion was supported by the observation that the appearance of the 220,000 molecular weight band was also associated with the appearance of several smaller polypeptides.

As a further test that the precipitated bands were associated specifically with virus production, a series of immunoprecipitations were performed on the low-level producer 7744 and a number of nonproducer cell lines (Fig. 10). The same pattern of radioactive bands was observed with 7744 as with B95-8 (confirmed by comigration on the same gel; data not shown). As would be expected for a low-level producer, the amount of material precipitated was small; consequently, it was necessary to expose the film for 1 month to detect the specific bands, compared with 36 h with B95-8. No specifically precipitated bands were detected from any of the nonproducer cell lines, even after 1 month of exposure. The lines tested included RAJI, an EBV-positive nonproducer B cell line derived from an African Burkitt's lymphoma biopsy; JY, an EBV-positive nonproducer B cell line derived from normal peripheral lymphocytes by in vitro transformation; and RAMOS, an EBV-negative B cell line derived from a Burkitt's lymphoma biopsy.

**Immunoprecipitation of the membrane antigens from Triton X-100-solubilized plasma membranes before and after lectin affinity chromatography.** By using the $^{125}$I-labeled protein A assay it was shown (see above) that the soluble plasma membrane fraction contained the same antigenic determinants before and after purification by Ricin affinity chromatography. To confirm this, immunoprecipitation was performed on these two fractions (Fig. 11). It was found that the 140,000 molecular weight polypeptide, which was only a minor component in the Ricin purified material, was a major component in the unfractionated material. Thus, the EBV-membrane antigens consist of three major polypeptides, one of which (140,000 molecular weight) did not bind extensively to Ricin or lentil

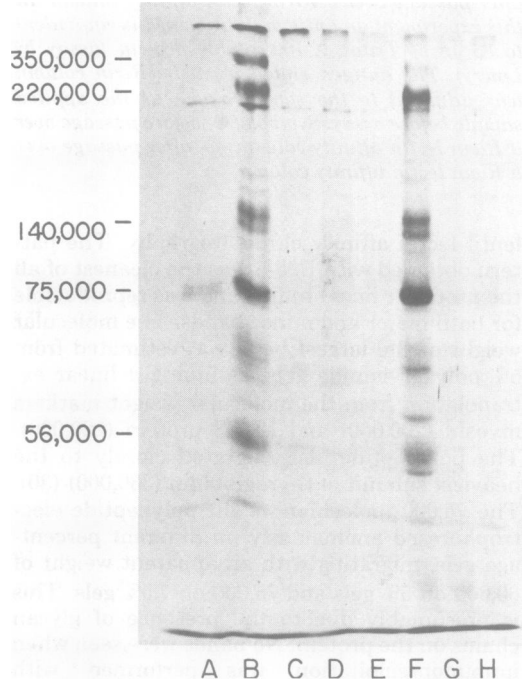


FIG. 9. *Immunoprecipitation of EBV membrane antigens from Ricin-purified P3HR-1 grown under stressed (P3HR-1 NONO) or normal (P3HR-1) conditions in the presence and absence of PAA. Note: this autoradiogram was exposed for 5 days. (A) P3HR-1 + preimmune serum; (B) as A + immune serum; (C) P3HR-1 + PAA + preimmune serum; (D) as C + immune serum; (E) P3HR-1 NONO + preimmune serum; (F) as E + immune serum; (G) P3HR-1 NONO + PAA + preimmune serum; (H) as G + immune serum.*

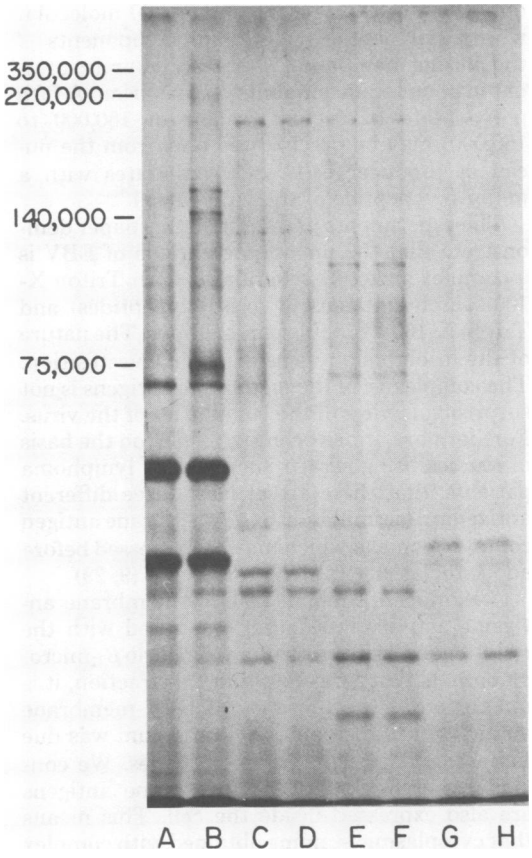


FIG. 10. *Immunoprecipitation of EBV membrane antigens from Ricin-purified membrane proteins of EBV-producer, nonproducer, and EBV-negative cell lines. Note: this autoradiogram was exposed for 1 month. (A) EBV-positive producer cell line 7744 + preimmune serum; (B) as A + immune serum; (C) EBV-positive nonproducer cell line RAJI + preimmune serum; (D) as C + immune serum; (E) EBV-positive nonproducer cell line JY + preimmune serum; (F) as E + immune serum; (G) EBV-negative cell line RAMOS + preimmune serum; (H) as G + immune serum.*

affinity columns and was not detected by the $^{125}$I-labeled protein A binding assay.

## DISCUSSION

In this paper data are presented on the purification and characterization of the EBV membrane antigen complex. The antigenic complex consists of three major polypeptides with molecular weights of 350,000, 140,000, and 75,000 plus several minor components, particularly of molecular weights of 220,000 and 56,000 (molecular weights are given for 7.5% SDS-PAGE). The 350,000 and 75,000 molecular weight components bind to Ricin and lentil lectin affinity columns. All of the polypeptides may be specifically precipitated from all the producer cell lines studied (P3HR-1, P3HR-1 NONO, B95-8, and 7744). This is particularly important considering the diverse origins of these cell lines. P3HR-1 is a subline derived from an African Burkitt's lymphoma which produces superinfecting but not transforming virus (16), B95-8 is a cell line derived by in vitro transformation of marmoset lymphocytes with EBV obtained from a patient with infectious mononucleosis (21), and 7744 is a subline of QIMR-WILL which was derived from a patient with myeloblastic leukemia (24). Both B95-8 and 7744 produce trans-

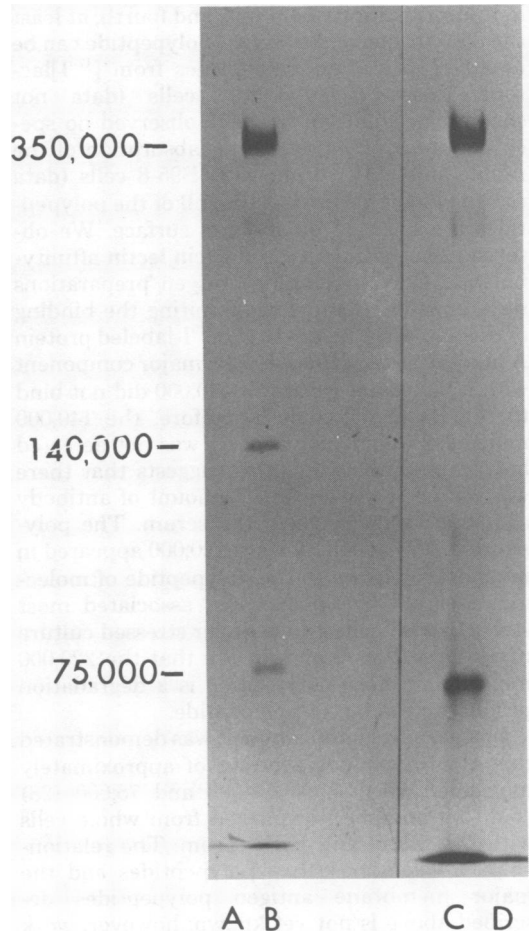


FIG. 11. *Immunoprecipitation of EBV membrane antigens from purified B95-8 plasma membranes before and after purification by Ricin affinity chromatography. (A) Whole Triton X-100-soluble plasma membrane proteins + preimmune serum; (B) as A + immune serum; (C) soluble plasma proteins purified by Ricin affinity chromatography + immune serum; (D) as C + preimmune serum. Note: these samples were electrophoresed in a 5% SDS gel.*

forming EBV. None of the polypeptides could be immunoprecipitated from nonproducer cell lines or from producer cell lines treated with phosphonoacetic acid at doses sufficient to inhibit late viral functions without affecting cellular growth (10, 21, 28). Thus, the immunoprecipitated polypeptides are truly late, i.e., post-viral DNA synthesis (26), EBV-associated polypeptides. That these polypeptides are genuine membrane proteins is indicated from several lines of evidence: first, they are found associated with a highly purified plasma membrane fraction; second, they are solubilized by the nonionic detergent Triton X-100; third, they are glycoproteins as judged by their binding to Ricin and lentil lectin affinity columns; and fourth, at least the 350,000 molecular weight polypeptide can be detected in immunoprecipitates from [$^{125}$I]lactoperoxidase-labeled whole cells (data not shown). In addition, we have observed no specific immunoprecipitate after absorption of the rabbit anti-EBV serum with B95-8 cells (data not shown). This implies that all of the polypeptides are exposed on the cell surface. We observed that both lentil and Ricin lectin affinity-purified EBV membrane antigen preparations were equally effective at inhibiting the binding of the anti-EBV serum in the $^{125}$I-labeled protein A binding assay. However, the major component with a molecular weight of 140,000 did not bind to the lectin columns. Therefore, the 140,000 molecular weight polypeptide was not detected by the binding assay. This suggests that there was only a relatively small amount of antibody against this molecule in the serum. The polypeptide of molecular weight 220,000 appeared in inverse proportion to the polypeptide of molecular weight 350,000 and was associated most strongly with cells grown under stressed culture conditions. This may indicate that the 220,000 molecular weight polypeptide is a degradation product of the larger polypeptide.

In a previous publication, it was demonstrated that two major polypeptides of approximately molecular weights of 150,000 and 75,000 (28) could be immunoprecipitated from whole cells with the rabbit anti-EBV serum. The relationship between these two polypeptides and the major membrane antigen polypeptides described above is not yet known; however, work under way at present in this laboratory suggests that the viral envelope contains at least four major polypeptides which are immunoprecipitated by the rabbit anti-EBV serum. These polypeptides have molecular weights of 350,000, 140,000, 75,000, and 56,000, and they comigrate with the equivalent bands precipitated from the plasma membranes of producer cells. However, only the 350,000, 140,000, and 75,000 molecular weight polypeptides are major components of the plasma membrane, whereas all four are present in about equal amounts on the virion. A fifth polypeptide (molecular weight of 150,000 to 160,000) may be precipitated only from the nuclei of producer cells and comigrates with a major polypeptide of the viral capsid.

The experiments presented in this paper demonstrate that the membrane antigen of EBV is a complex structure, containing both Triton X-100-soluble antigens (3 to 5 polypeptides) and Triton X-100-insoluble polypeptides. The nature of the insoluble antigens is under investigation. The complexity of the membrane antigens is not surprising in view of the complexity of the virus. Furthermore, it has been suggested, on the basis of studies using sera from Burkitt's lymphoma patients, that there are at least three different antigenic determinants in the membrane antigen complex, some of which may be expressed before and some after viral DNA synthesis (9, 29).

A significant amount of EBV-membrane antigenic activity was found associated with the crude endoplasmic reticulum. Since no $\beta_2$-microglobulin activity was found in this fraction, it is unlikely that the presence of EBV-membrane antigens in the endoplasmic reticulum was due to contaminating plasma membranes. We conclude, therefore, that the membrane antigens are also expressed inside the cell. This means that cytoplasmic staining obtained with complex human sera is probably due to a combination of antimembrane (expressed on the endoplasmic reticulum?) and antinucleocapsid reactivities. We are at present studying the subcellular distribution of the molecules identified in this paper as well as other virus-specific polypeptides to understand better the structure of the virus and the way it is assembled.

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## LITERATURE CITED

1. **Baron, D., and J. L. Strominger.** 1978. Partial purification and properties of the Epstein-Barr virus associated nuclear antigen. J. Biol. Chem. **253**:2875-2881.
2. **Bennet, T. P.** 1967. Membrane filtration for determining protein in the presence of interfering substances. Nature (London) **213**:1131-1132.
3. **Cone, R. E., and W. C. Brown.** 1967. Isolation of membrane associated immunoglobulins from T lymphocytes by non-ionic detergents. Immunochemistry **13**:571-581.
4. **Crumpton, M. J., and D. Snary.** 1974. Preparation and

properties of lymphocyte plasma membranes, p. 27-56. *In* G. C. Ada (ed.), Contemporary topics in molecular immunology, vol. 3. Plenum Press, N.Y.

5. **de-Thé, G., A. Gese, N. E. Day, P. M. Tukei, E. H. Williams, D. P. Beri, P. G. Smith, A. G. Dean, G. W. Bornkamm, P. Feorino, and W. Henle.** 1979. Epidemiological evidence for causal relationship between Epstein-Barr virus and Burkitt's lymphomas from Ugandan prospective study. Nature (London) **274**: 756-761.
6. **Dulley, J. R., and P. A. Grieve.** 1975. A simple technique for eliminating interference by detergents in the Lowry Method of protein determination. Anal. Biochem. **64**: 136-141.
7. **Epstein, M. A.** 1976. Implications of a vaccine for the prevention of Epstein-Barr virus infection: ethical and logistic considerations. Cancer Res. **36**:711-714.
8. **Epstein, M. A., B. G. Achong, and Y. M. Barr.** 1964. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet **i**:702-703.
9. **Ernberg, I., G. Klein, F. M. Kourilsky, and D. Silvestre.** 1974. Differentiation between early and late membrane antigen on human lymphoblastoid cell lines infected with Epstein-Barr virus. I. Immunofluorescence. J. Natl. Cancer Inst. **53**:61-65.
10. **Granlund, D. J., and G. R. Pearson.** 1977. Membrane antigen expression in Epstein-Barr virus-infected Raji cells in the presence of phosphonoacetic acid. Virology **83**:217-220.
11. **Greenwood, F. C., W. M. Hunter, and J. S. Glover.** 1963. The preparation of $^{131}$I-labelled human growth hormone of high specific radioactivity. Biochem. J. **89**: 114-123.
12. **Henle, G., W. Henle, P. Clifford, V. Diehl, G. W. Kafuko, B. G. Kurya, G. Klein, R. H. Morrow, G. M. R. Munube, P. Pike, P. M. Tukei, and J. L. Ziegler.** 1969. Antibodies to Epstein-Barr virus in Burkitt's lymphoma and control groups. J. Natl. Cancer Inst. **43**:1147-1157.
13. **Henle, W., V. Diehl, G. Kohn, H. zur Hausen, and G. Henle.** 1967. Herpes type virus and chromosome marker in normal leukocytes after growth with irradiated Burkitt cells. Science **157**:1064-1065.
14. **Henle, G., W. Henle, and V. Diehl.** 1968. Relation of Burkitt's tumor-associated herpes-type virus to infectious mononucleosis. Proc. Natl. Acad. Sci. U.S.A. **59**: 94-101.
15. **Henle, G., W. Henle, G. Klein, P. Gunven, P. Clifford, R. M. Morrow, and J. L. Ziegler.** 1971. Antibodies to early Epstein-Barr virus induced antigens in Burkitt's lymphomas. J. Natl. Cancer Inst. **46**:861-871.
16. **Hinuma, Y., and J. T. Grace.** 1967. Cloning of immunoglobulin producing human leukemic and lymphoma cells in long term cultures. Proc. Soc. Exp. Bio. (N.Y.) **126**:107-111.
17. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein-A antibody absorbent. J. Immunol. **1115**:1617-1624.
18. **Luka, J., T. Lindahl, and G. Klein.** 1978. Purification of the Epstein-Barr virus-determined nuclear antigen from Epstein-Barr virus-transformed human lymphoid cell lines. J. Virol. **27**:604-611.
19. **Matsuo, T., N. Hibi, S. Nishi, H. Hirai, and T. Osato.** 1978. Studies on Epstein-Barr virus related antigens. III. Purification of the virus determined nuclear antigen (EBNA) from non-producer Raji cells. Int. J. Cancer **22**:747-752.
20. **Miller, G., J. C. Niederman, and L. Andrews.** 1973. Prolonged oropharyngeal excretion of Epstein-Barr virus after infectious mononucleosis. N. Eng. J. Med. **288**: 229-232.
21. **Miller, G., T. Shope, H. Lisco, D. Stitt, and H. Lipman.** 1972. Epstein-Barr virus: transformation, cytopathic changes and viral antigens in squirrel monkey and marmoset leukocytes. Proc. Natl. Acad. Sci. U.S.A. **69**:383-387.
22. **Nyormoi, O., D. A. Thorley-Lawson, J. Elkington, and J. L. Strominger.** 1976. Differential effects of phosphonoacetic acid on the expression of Epstein-Barr viral antigens and virus production. Proc. Natl. Acad. Sci. U.S.A. **73**:1745-1748.
23. **Pearson, G. R., and L. F. Qualtiere.** 1978. Papain solubilization of the Epstein-Barr virus-induced membrane antigen. J. Virol. **28**:344-351.
24. **Pope, J. H.** 1968. Establishment of cell lines from Australian leukaemic patients: presence of a herpes like virus. Austral. J. Exp. Biol. Med. Sci. **46**:643-645.
25. **Reedman, B. M., and G. Klein.** 1973. Cellular localization of an Epstein-Barr virus (EBV)-associated complement fixing antigen in producer and non-producer lymphoblastoid cell lines. Int. J. Cancer **11**:499-520.
26. **Summers, W. G., and G. Klein.** 1976. Inhibition of Epstein-Barr virus DNA synthesis and late gene suppression by phosphonoacetic acid. J. Virol. **18**:151-155.
27. **Svedmyr, A., A. Demissie, G. Klein, and P. Clifford.** 1970. Antibody patterns in different human sera against intracellular and membrane-antigen complexes associated with Epstein-Barr virus. J. Natl. Cancer Inst. **44**: 595-610.
28. **Thorley-Lawson, D. A.** 1979. Characterization of cross-reacting antigens on the Epstein-Barr virus envelope and plasma membranes of producer cells. Cell **16**:33-42.
29. **Thorley-Lawson, D. A., and N. M. Green.** 1975. Separation and characterization of tryptic fragments from the adenosine triphosphatase of sarcoplasmic reticulum. Eur. J. Biochem. **59**:193-200.
30. **van der Walt, B., B. Kotze, P. P. van Iaarsveld, and H. Edelhoch.** 1978. Evidence that thyroglobulin contains nonidentical half molecule subunits. J. Biol. Chem. **253**:1853-1858.
31. **Warburg, O., and W. Christian.** 1941. Isolierung and kristallisation de garungsferments enolase. Biochem. Z. **310**:384-421.
32. **Weinberg, A., and Y. Becker.** 1969. Studies on EB virus of Burkitt's lymphoblasts. Virology **39**:312-321.
33. **zur Hausen, H., V. Diehl, H. Wolf, H. Schulte-Holthausen, and U. Schnider.** 1972. Occurrence of Epstein-Barr virus genomes in human lymphoblastoid cell lines. Nature (London) New Biol. **237**:189-190.