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Two Large Virion Envelope Glycoproteins Mediate Epstein-Barr Virus Binding to Receptor-Positive Cells

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The four major Epstein-Barr virion envelope components were separated by column chromatography and reconstituted into artificial liposomes. These liposomes were tested for their ability to bind selectively to Epstein-Barr virus receptor-positive cells. Only when the two high-molecular-weight glycoproteins, VE1 and VE2, were present together was a stable binding complex formed. The addition of the other virion envelope components did not increase the levels of binding. This binding was inhibited by unlabeled viable virions and by neutralizing antisera, which recognized the two components. Adsorption of viable virus was also eliminated by the antisera. The enzyme susceptibility pattern of the cell-liposome interaction is similar to that of the virus-cell interaction, thus confirming the specificity of the binding site. A model for Epstein-Barr virus binding in which VE1 and VE2 coordinately recognize the same binding site is presented.

Epstein-Barr (EB) virus causes infectious mononucleosis and is closely associated with two human malignant tumors, African Burkitt's lymphoma and undifferentiated nasopharyngeal carcinoma (for review see reference 7). Two of these diseases are lymphoproliferative disorders of B-cell origin. Since virus receptors have been found only on B-lymphocytes of human and certain nonhuman primate origin, the surface components of EB virus play a major role in restricting the host range of the virus.

More recently, it has been shown that by artificially bypassing the membrane barrier EB virus genes are expressed in a wide range of naturally nonpermissive hosts (10, 35), thus demonstrating the crucial role of the EB virus receptor in control of infection. Although the binding specificity of the EB virions has been demonstrated (20, 36), very little is known about the molecular aspects. Other herpes-group viruses have been more extensively studied than EB virus, but little is known about the specific binding moieties of the virions (27, 30).

Structural polypeptides of semipurified EB virions have been investigated (4, 5). Comparison of the protein patterns of enveloped virions and Nonidet P-40-treated virions suggests that 10 of 34 structural proteins are envelope associated. The EB virus specificity of these proteins was not examined. Recent investigations concerning the EB membrane antigens (for review see reference 7) were performed by immunoprecipitation of virus-producing cells (16, 25, 26, 28, 31, 33) and virion particles (6). Despite molecular-weight discrepancies due to different polyacrylamide gel electrophoresis (PAGE) conditions and markers, a consistent pattern emerges. Four to five polypeptides ranging from 80×10^3 to 350×10^3 daltons comprise the membrane antigen complex on the cells. Membrane antigen components of similar size have been observed on the virion envelopes (6, 29). Previously, we found that the virus envelope contains four major proteins and a fifth minor one which was neither labeled by surface labelings nor detected by immunoprecipitation of solubilized virions (unpublished data). To avoid confusion over molecular-weight sizing, we designated these protein moieties VE1 through VE5, in order of decreasing molecular weight. VE4 is the minor component. Furthermore, we were able to separate these components by molecular-weight column chromatography.

Utilizing the purified individual virion envelope proteins reconstituted in artificial liposomes, we have investigated the role of each as it pertains to virion binding to the EB virus receptor-positive cells. The two high-molecular-weight components, VE1 and VE2, were found to be responsible for this property, both being needed for stable adherence of virus to cell. Neutralizing antisera which recognized these two components were also used in studies of this phenomenon. These tests revealed that the antisera directly inhibit virion binding to cells. This mode of action is responsible for virus neutral-

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ization in vitro and may be indicative of the in vivo immune response.

MATERIALS AND METHODS

Reagents and radioisotopes. Dextran T-10, Sepharose 6B, and Sephacryl S-300 were purchased from Pharmacia Chemicals, Sweden. Triton X-100, sodium dodecyl sulfate (SDS), phosphatidylcholine (lecithin), phosphatidylserine, and cholesterol were obtained from Sigma Chemical Co. L-[^{35}S]methionine (800 Ci/mmol), [methyl-^{3}H]thymidine (60 to 80 Ci/mmol), and Na^{125}I were purchased from New England Nuclear. ^{14}C-methylated proteins, New England Nuclear products, were used as molecular-weight markers for SDS-PAGE: phosphorylase b (92,500), bovine serum albumin (69,000), ovalbumin (46,000), carbonic anhydrase (30,000), and cytochrome *c* (12,500). Unlabeled high-molecular-weight markers consisting of porcine thyroglobulin (669,000; subunit, 330,000), ferritin (440,000; subunit, 18,500), catalase (232,000; subunit, 60,000), lactate dehydrogenase (140,000; subunit, 36,000), and bovine serum albumin (67,000) were purchased from Pharmacia. Myosin (220,000) was a gift from J. Luka in our department.

Cells. Raji (8, 19) and Daudi (17, 19) are EB virus-carrying, receptor-positive, cell lines derived from Burkitt's lymphoma. YAC (18), a mouse T-cell line, 1301 (9), a human T-cell line, and K562 (1, 37), a human erythroid-derived line, were used interchangeably as the EB virus receptor-negative control targets (35). All cell culturing was performed in RPMI 1640 medium (GIBCO Laboratories, Scotland) supplemented with 10% fetal calf serum and antibiotics (200 U of penicillin and 100 μg of streptomycin per ml) at 37°C, 5% CO_2, and 60% humidity.

Virus purification and in vivo labeling. Virus-producing B95-8 cells were grown as suspension cultures for 1 week from an initial density of 3×10^5 cells per ml. The virus was metabolically labeled by adding [^{3}H]thymidine (2 μCi/ml) or [^{35}S]methionine (40 μCi/ml) to the cell cultures on the first and fourth days. After the culture supernatants were harvested, the virus was purified by the procedure of Dolyniuk et al. (4) as modified previously by us (20.) Briefly, virus pelleted by centrifuging at 13,300 × *g* for 120 min was ultracentrifuged on successive 5 to 30% and 10 to 30% dextran gradients for 1 h each at 21,000 rpm in an SW 27.1 rotor (Beckman Instruments, Inc.). The dextran was dissolved in 0.5 mM phosphate buffer (pH 8.0) containing 1 mM EDTA and 0.02% NaN_3 (gradient buffer). As demonstrated earlier (20), the radioactivity and infectivity peaks of both gradients coincided at 17 to 18% dextran (Fig. 1A, C, E). The 17 to 18% dextran fractions of the first gradient were diluted twofold with gradient buffer before they were applied to the second gradient. The peak fractions of the second gradient were collected for use.

Virus was separated from dextran by column chromatography on Sepharose 6B in a column (0.7 by 30 cm) equilibrated with gradient buffer. Virion particles were collected in the void volume fractions and pelleted by centrifugation at 15,000 × *g* for 1 h. The pelleted virus was used for in vitro labeling and envelope solubilization.

Radioiodination of virion particles. The virus was iodinated with 1 mCi of Na^{125}I by one of two procedures, the chloramine-T method (24) or with lactoperoxidase-peroxide (23). Approximately 100 μg of dextran-free virus, determined by the method of Lowry et al. (22), was suspended in 50 μl of phosphate-buffered saline (0.1 M phosphate buffer, pH 8.0, containing 0.15 M NaCl). In the chloramine-T method, the reaction was initiated by twice adding 10 μg of chloramine-T at a 1-min interval to the virus and Na^{125}I mixture. After a 2-min incubation, the reaction was quenched by introducing 20 μg of $Na_2S_2O_5$ dissolved in phosphate buffer containing 0.15 M NaI. Lactoperoxidase labeling was initiated by the addition of 10 μg of lactoperoxidase in phosphate-buffered saline and 10 μl of 0.3% H_2O_2 to the virus and isotope mixture. The reaction was continued by adding 5 μl of peroxide three times at 5-min intervals and was terminated by introducing 200 μl of phosphate buffer containing 0.15 M NaI. The labeled virus was separated from free iodine and chemicals by use of a Sepharose 6B column and was repurified on successive 5 to 30% and 10 to 30% dextran gradients, as above.

Detergent solubilization of virus. A sample of the dextran-free virus pellets was suspended (by gentle blending in a Vortex mixer) in 500 μl of 1% Triton X-100 in NET buffer (10 mM Tris-hydrochloride buffer, pH 8.0, containing 2 mM EDTA, 0.15 m NaCl, and 0.02% NaN_3) and was incubated for 20 min at room temperature. The mixture was layered onto a 5 to 30% dextran gradient containing 0.1% Triton X-100 and centrifuged at 21,000 rpm for 60 min in an SW 27.1 rotor. In the later studies, the Triton X-100–treated virus was centrifuged at 40,000 × *g* for 1 h to pellet the nucleocapsid.

Virion envelope component separation by column chromatography. Sephacryl S-300 packed in a column (1.2 by 90 cm) equilibrated and eluted with NET buffer containing 0.05% Nonidet P-40 and 0.05% deoxycholate (Sigma); 0.5-ml fractions were collected. The column was calibrated with unlabeled and radioiodinated high-molecular-weight protein markers (Pharmacia).

Reconstitution of virion proteins in liposomes. Phosphatidylcholine, cholesterol, and phosphatidylserine, in the mass ratio of 7:2:1, were employed in the construction of artificial liposomes (2, 11, 15). The lipids, dissolved in chloroform or diethylether, or both, were dried under streaming N_2 gas. The mixture was then dissolved in 10 mM Tris-hydrochloride (pH 8.5) containing 2 mM $CaCl_2$ and 2 mM $MgCl_2$ (reconstitution buffer) and 2% deoxycholate and was sonicated. Protein samples to be reconstituted in liposomes, in reconstitution buffer and 2% deoxycholate, were added to the lipid stock to a final concentration of 2 mg/ml of lipids. This was dialyzed for 48 h against 1,000 volumes of reconstitution buffer containing 0.02% NaN_3 and 1% bovine serum albumin. The liposomes were then pelleted at 100,000 × *g* for 45 min for non-nucleocapsid liposomes and 30,000 × *g* for 45 min for the nucleocapsid-containing liposomes.

Binding assay. As described earlier (20), we used a direct radiobinding assay. Briefly, 1,000 cpm of thymidine-labeled material or 5,000 cpm of iodinated sample were incubated with 4×10^6 cells in 1 ml of 1% bovine serum albumin in balanced salt solution. After 30 min of incubation of 20°C, the cell suspension was applied to the top of 1 ml of 10% sucrose in balanced salt solution, centrifuged for 60 s at 9,000 × *g*, and washed

once with 1% bovine serum albumin in balanced salt solution. The radioactivity of the pellet and supernatant was measured separately.

Antisera. Origins, specificities, and references concerning the antisera used are listed in Table 1. The EB virus-positive serum was a pool of two Burkitt's lymphoma and four nasopharyngeal carcinoma patients, all with high titers to EB virus-determined antigens (unpublished data). EB virus-negative serum was from a healthy, seronegative donor, devoid of detectable titers to EB virus antigens (unpublished data). The GJH sera were from the ascites fluids of a murine monoclonal, directed against VE1, and were the very kind gift of Gary J. Hoffman (14). David Thorley-Lawson and Clark Edson kindly donated the DTL serum, a rabbit monospecific serum which recognizes both VE1 and VE2 (34).

RESULTS

Specificity of radiolabeling and virion binding. We previously investigated the specificity and purity of the isotope labeling procedures on the virions (unpublished data). These results are summarized in Fig. 1, 2, and 3. In short, the thymidine label was found to be associated with the virion nucleocapsid which appeared at 24 to 26% dextran after Triton treatment of the virion (Fig. 1B), and the iodinated material was detected in the soluble proteins (Fig. 1F). Proteins metabolically labeled by the addition of [^{35}S]methionine were associated with both the nucleocapsid and soluble fractions (Fig. 1D).

The thymidine-labeled material was examined by DNA extraction, restriction endonuclease treatment, and blot hybridization, and was found to be more than 95% pure, nondegraded EB virus DNA (A. Saemundsen, personal communication). Iodinated and methionine-labeled components were precipitated with trichloroacetic acid and immunoprecipitated to analyze their labeling distribution. [^{35}S]methionine presented numerous bands, four of which were associated with the soluble fraction. The iodinated proteins (Fig. 2) were identical to these four. Sugar labeling, metabolically but not extrinsically, revealed a fifth, minor, envelope-associated protein (unpublished data). The approximate molecular weight and suggested distribution of the envelope components are presented in Table 2.

The labeled virions were tested for their ability to adsorb specifically to receptor-positive cells (Fig. 3) (20). Ninety percent of the thymidine label was associated with virions which adsorbed to Raji cells. The percentages for methionine and iodinated virions were 70 and 40%, respectively. These levels are rough indicators of label purity, as discussed previously (20; unpublished data). In all labels the negative control cells bound less than 7% of the gross counts added.

Separation of envelope proteins by column chromatography. Solubilized, iodinated material was run in a Sephacryl S-300 column precalibrated with a series of nonlabeled and ^{125}I-labeled proteins (Fig. 4). One-milliliter fractions were collected and counted, with the different peaks being pooled. These peak fractions were precipitated with trichloroacetic acid and analyzed by SDS-PAGE, with the autoradiograms being scanned by a scanning densitometer. The tracings revealed separation of the four major envelope proteins (Fig. 5). VE4, which is poorly iodinated (unpublished data), was not detected. In later studies, we performed the iodination after solubilization of the virion and isolation of the envelope components. By this procedure we obtained a 10-fold greater labeling efficiency but

TABLE 1. Serum specificities

Serum[a]	Origin[b]	VCA titer[c]	EA titer[c]	EBNA titer[c]	VE specificity[d]	Neutralization potential[e]
EB virus-positive (U)	Human BL	5,120	320	2,040	VE1, 2, 3, 4, 5	Yes
	Human BL	5,120	160	10		
	Human NPC	1,280	160	160		
	Human NPC	1,280	320	10		
	Human NPC	2,560	320	640		
	Human NPC	1,280	640	40		
EB virus-negative (U)	Human, healthy	—	—	—	None	No
GJH (14)	Murine monoclonal				VE1	Yes
DTL (34)	Rabbit				VE1, 2	Yes

[a] Numbers in parentheses are references. (U) Unpublished data.
[b] BL, Burkitt's lymphoma; NPC, nasopharyngeal carcinoma.
[c] Antibody titers against the EB virus-associated antigens are expressed as the reciprocal of the endpoint dilution as determined by W. Henle and G. Henle in the course of an earlier investigation. VCA, Virus capsid antigen; EA, early antigen; EBNA, EB nuclear antigens.
[d] Virion envelope components immunoprecipitated by the sera; see references.
[e] Determined by inhibition of EA induction in Raji cells as described by Coope et al. (3).

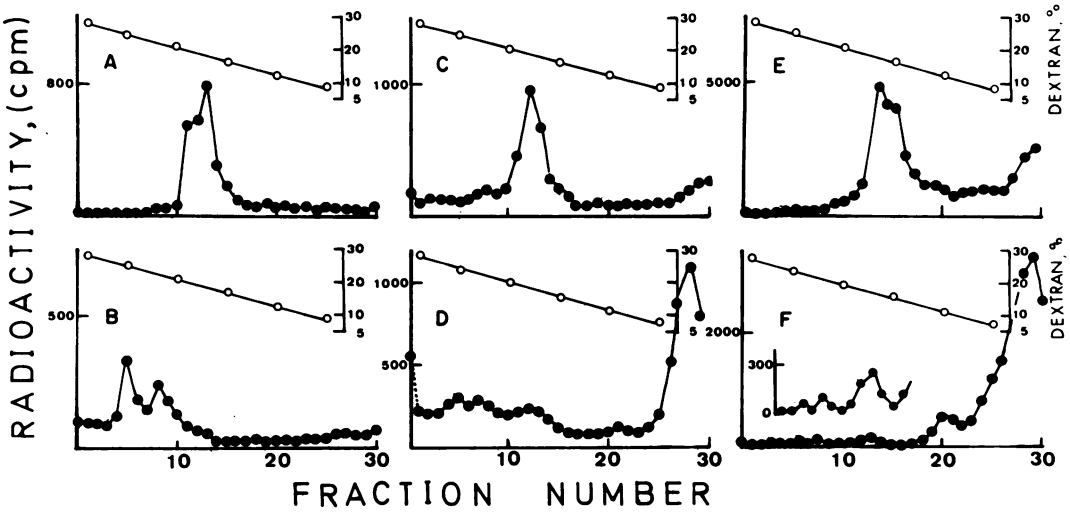

FIG. 1. Gradient profiles of labeled virions. Virions labeled with [*methyl*-^{3}H]thymidine (A and B), [^{35}S]methionine (C and D), or ^{125}I (E and F) were ultracentrifuged first in a 5 to 30% dextran gradient and then a 10 to 30% gradient. The radioactivity profile of the second gradient is shown on the top row (A, C, E). Samples of the peak fractions from the second gradient, diluted in gradient buffer, were pelleted at 15,000 rpm in an SW 27.1 rotor and then suspended in NET buffer containing 1% Triton X-100. After 20 min of incubation at room temperature, the virions were layered on a 5 to 30% dextran gradient containing 0.1% Triton and were ultracentrifuged as in the first gradients. Fractions were collected and assayed for radioactivity, and the profiles are presented in B, D, and F.

were still unable to detect VE4. As such, VE4 was not included in our assay system.

Five liters of virus-containing supernatants yielded approximately 1 μg of pure component after column separation. Trace amounts of these proteins were incorporated into liposomes as described. About half of the radiolabel was pelleted with the liposomes. As a result of the extremely small amounts of protein, we were unable to directly ascertain the nature of the proteins in the liposomes and, therefore, relied on indirect proof of liposome composition.

Inhibition of EB virus binding by virion components. Nonlabeled virion envelope components were purified in parallel with the iodinated preparations and were individually incorporated into liposomes. These liposomes were then tested for their ability to block adsorption of labeled virions to EB virus receptor-positive cells (Fig. 6). Both of the higher-molecular-weight envelope components demonstrated an inhibitory effect, whereas VE3 and VE5 did not. We investigated the time kinetics of the inhibition caused by VE2 (Fig. 7) because none of the individual envelope components presented stable binding (Fig. 8). Nonlabeled VE2, 10^{-13} mol, was added to Raji cells in the presence of iodinated virion and sampled at various time points to determine the inhibitory effect of the protein. The difference between the test and control populations was maximal at the earliest stages. By 1 h this difference had vanished, suggesting that VE2 and VE1 (data not shown) interfere with virion binding via a competitive weak interaction between the EB virus receptor and the individual components, whereas the virion adsorbed to receptor is less easily displaced.

Liposomes containing both VE1 and VE2 stably adsorb to EB virus receptor-positive cells. Individual iodinated virion proteins were incorporated into liposomes and assayed for ability to specifically bind to receptor-positive cells. As is shown in Fig. 8, the liposomes containing individual virion envelope components did not display this property, whereas the liposomes containing both high-molecular-weight components did. Addition of 10 μl of GJH or DTL sera to the liposomes for 30 min prior to coincubation with cells prevented this binding, whereas the same quantity of EB virus-negative sera did not have any effect (data not shown). These results strongly suggest that the observed binding is mediated by the biologically active sites of VE1 and VE2.

As a further control of the site for liposome adsorption, a competition experiment was performed in which unlabeled virions were used. An excess of unlabeled virions, corresponding to approximately 10^{-12} mol of virion as estimated by protein determination (22), was diluted and coincubated with liposomes and cells. Ex-

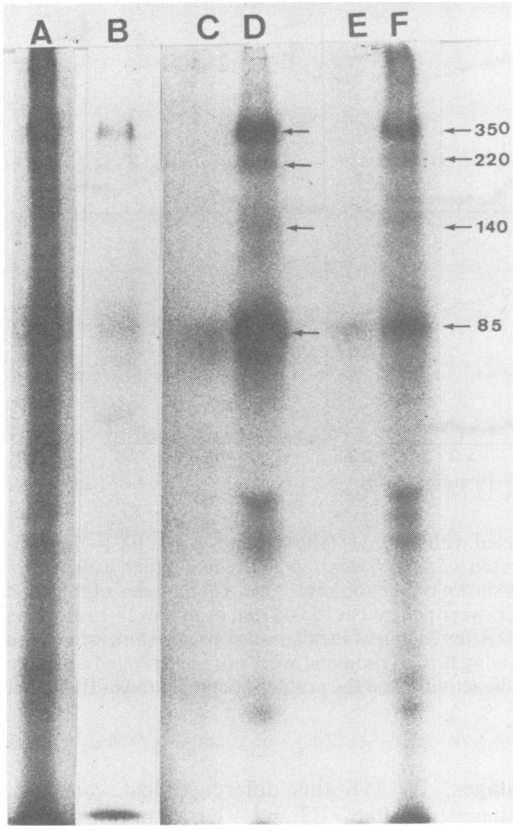

FIG. 2. Polypeptide pattern of ^{125}I-labeled virions. Virions were labeled with ^{125}I as described. The purified virions were then treated with Triton X-100 (1% for 20 min) and ultracentrifuged on a dextran gradient to separate nucleocapsids from the soluble material. Different samples were precipitated by either 10% trichloroacetic acid or immune sera (unpublished data). The precipitated material was dissolved in electrophoresis sample buffer containing SDS and 5% β-mercaptoethanol (21). SDS-PAGE was performed on 5 to 15% continuous gradient slab gels (21). Gels containing the iodinated material were directly dried and provided for autoradiography using intensifying screens (Eastman Kodak Co.) (32). The numbers represent the approximate molecular weights (in thousands) as determined by a series of molecular-weight markers run in parallel. (A) Whole virions treated with trichloroacetic acid, (B) envelope material precipitated with trichloroacetic acid, (C) envelope material precipitated by EB virus-negative sera, (D) envelopes precipitated by EB virus-positive sera, (E) whole virions and EB virus-negative sera, and (F) whole virions and EB virus-positive sera.

cess virus completely inhibited liposome binding (Fig. 9). Inhibition of liposome adsorption was thus achieved by both unlabeled virus and specific antisera, confirming that liposome binding is not artifactual.

Adsorption is mediated only by VE1 and VE2. EB virus nucleocapsids were isolated from the 24 to 26% dextran fraction of Triton-treated, thymidine-labeled virions (Fig. 1B) and incorporated into liposomes along with nonlabeled envelope proteins. Because the label was protected from direct contact with the microenvironment, these liposomes could be employed to determine whether the liposomes are internalized by the cells. Liposomes containing the nucleocapsid and different combinations of the four major envelope components were adsorbed to receptor-positive and -negative cells for 2 h at 37°C, a temperature permissive for viral internalization (Fig. 10). After this incubation, a sample of each liposome-cell mixture was trypsinized briefly to remove exposed surface structures. The trypsin treatment removed more than 80% of the specifically bound label in all cases. Because bare nucleocapsids demonstrate 30% of the binding level of virions (20), a liposome containing only the nucleocapsid was reconstituted and tested in parallel (Fig. 10E). This sample did not show any specific adsorption to EB virus receptor-positive cells, suggesting that the nucleocapsid was enveloped by the artificial liposomes.

Enzyme treatment of receptor site for virion components suggests identity with virion binding site. We have previously shown that the EB virus receptor is neuraminidase resistant but trypsin sensitive (20). To demonstrate a correspondence between the liposome target structure and that of the virion as regards enzyme susceptibility, we treated Raji cells with neuraminidase or trypsin as described in the Fig. 11 legend. Adsorption by liposomes containing virion envelope components was not disturbed by neuraminidase, but pretreating the cells with trypsin eliminated specific binding, in agreement with the findings for the viable virions. The negative controls were also enzymatically treated to determine whether the high level of adsorption by liposomes containing virion envelope components observed after neuraminidase treatment is due to the generation of a "sticky surface." Such a situation is not observed with the negative cells. Additionally, a liposome containing all four envelope components was tested in parallel to assess the difference in binding between such a liposome and one containing only VE1 and VE2. No difference between the two was observed. The slight increase in liposome binding seen after the neuraminidase treatment is not significant, being within one standard error of the nontreated control.

DISCUSSION

Individual EB virion envelope components were separated under nondenaturing conditions to identify the proteins responsible for binding.

TABLE 2. EB virus envelope components

Designation[a]	Approx mol wt[b]	Labeling preference[c]		Suggested location
VE1[d]	350,000	I	+++	Envelope-associated, externally exposed
		M	++	
		S	+++	
VE2[d]	220,000	I	+++	Envelope-associated, externally exposed
		M	+	
		S	++	
VE3	140,000	I	+	Envelope-associated, externally exposed
		M	+++	
		S	−	
VE4	130,000	I	−	Envelope-associated, nonexposed
		M	+	
		S	+	
VE5	85,000	I	+++	Envelope-associated, externally exposed
		M	++	
		S	++	

[a] Unpublished data.
[b] Determined on SDS-PAGE (unpublished data).
[c] Relative labeling strength of radioisotopes: I, ^{125}I by the chloramine-T method; M, metabolically by [^{35}S]methionine; S, various sugar residues both in vivo and in vitro (unpublished data). Symbols: +++, strong; ++, medium; +, weak; −, no labeling detected.
[d] The B95-8 virus substrain, which has a high 350/220 ratio, is examined here; the P3HR-1 substrain is discussed elsewhere (6).

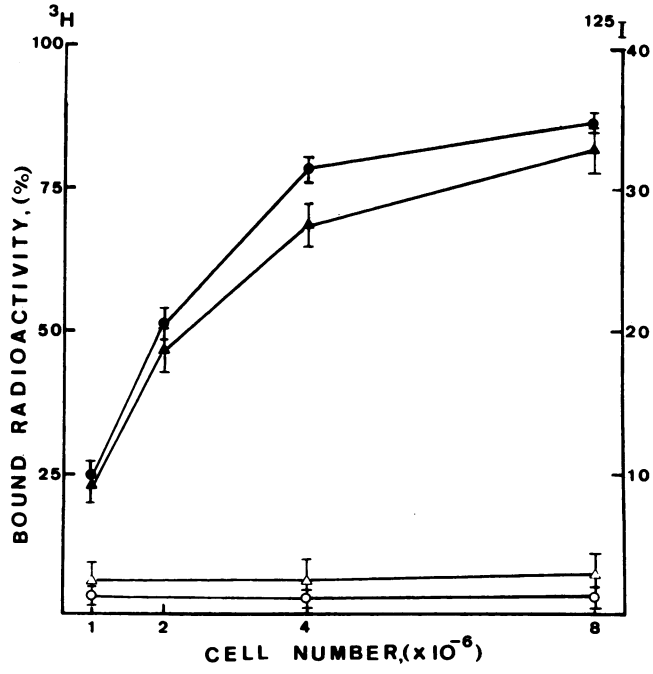

FIG. 3. Binding of radiolabeled virus to cells. Preparation of virus and binding assay are described in Materials and Methods. Closed and open markers represent the adsorbed radioactivity by receptor-positive Raji cells and receptor-negative 1301 or K562 cells, respectively. Circles represent the binding of 1,000 cpm of [^{3}H]thymidine-labeled virions and triangles, that of 5,000 cpm of iodinated virions.

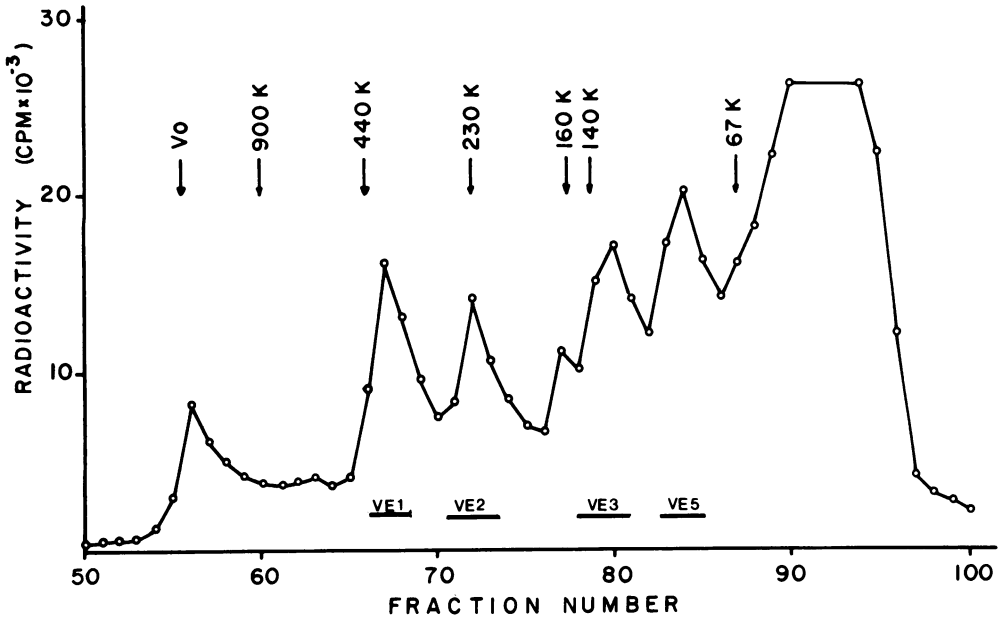

FIG. 4. Profile of virion envelope components on a Sephacryl S-300 column. Iodinated virions were treated with 1% Triton X-100 for 20 min and then centrifuged in a 5 to 30% dextran gradient to separate the soluble components from the nucleocapsids. The top fractions of the gradient (Fig. 1F) were collected and then chromatographed on a Sephacryl S-300 column (1.2 by 90 cm). The column was equilibrated and eluted with NET buffer containing 0.05% deoxycholate and 0.05% Nonidet P-40. One-milliliter fractions were collected and assayed for radioactivity. The column was pre- and postcalibrated with high-molecular-weight markers (Pharmacia, Sweden): immunoglobulin (900K), ferritin (440K), catalase (230K), immunoglobulin G (160K), lactate dehydrogenase (140K), and bovine serum albumin (67K). The collected fractions are indicated by the bars.

Unfortunately, these proteins indiscriminately adsorbed to all cells (data not shown), most probably as a result of hydrophobic interactions. A special orientation for binding was suggested, and therefore artificial liposomes were employed to reduce nonspecific adsorption of the proteins and to orient the proteins correctly in terms of hydrophilic-hydrophobic moieties. The purified individual components incorporated into liposomes presented low levels of background adsorption to EB virus receptor-negative cell lines. Liposomes bound specifically to receptor-positive cells only when both VE1 and VE2 were present. The other two envelope proteins did not seem to be involved in the initial stage of virus adsorption. Additionally, antisera directed against either of these two binding components inhibited binding of viable virions to target cells, further demonstrating the role of both glycoproteins in virus infection.

There are two possible sources of systematic error in our assays. These involve component purity and liposome composition. A number of precautions were instituted to control purity of the different envelope components. These proteins, though present in greater quantities on producer cells (6, 25, 28, 31, 33), were isolated from three or four gradient-purified virions. The disadvantage of paucity of material was compensated by the low number of Triton-soluble proteins in these preparations, which yielded only four different soluble, iodinated components (Fig. 2). These proteins were previously analyzed extensively by both trichloroacetic acid precipitation and immunoprecipitation (33; unpublished data). After column chromatography, narrow peaks were collected and purity was ascertained by trichloroacetic acid precipitation and SDS-PAGE (Fig. 5). The samples were quite clean and free from significant cross-contamination. As a result of these controls, only trace amounts of proteins were obtainable. Approximately half of the protein added to the lipid mixture was pelleted with the reconstituted liposomes, as determined by distribution of label. However, with trace amounts of proteins involved, it was impossible to monitor the liposomes' composition and component orientation directly. Rescue of specific proteins from liposomes (data not shown) did not indicate spatial

FIG. 5. Densitometer tracings of the individual envelope components. Peak fractions from the Sephacryl S-300 column (Fig. 4) were collected and precipitated with trichloroacetic acid. The iodinated material was electrophoresed in a 5 to 15% gradient slab SDS-polyacrylamide gel under reducing conditions. The gel was dried and processed as described in the legend for Fig. 2. The autoradiograph was evaluated with a scanning densitometer. Direction of migration is right to left. From top to bottom the tracings represent: fractions 66–69, fractions 71–73, fractions 78–81, and fractions 83–85. In later studies only fractions 66–68 were pooled for VE1, thus reducing the slight contamination of VE2 observed in the top tracing. The arrows designate the various envelope components as determined by markers run in parallel.

organization. With these limitations in mind, we relied on indirect indications of specificity and construction of the liposomes.

Both VE1 and VE2 inhibited viral binding, whereas the other two proteins did not. Because results of the binding assay performed in balanced salt solution with 1% bovine serum albumin or RPMI 1640 medium with 10% fetal calf serum were similar, the inhibitory effect was unlikely to be due to nonspecific protein or lipid interference of virion and receptor interaction. Experiments were designed to demonstrate that the liposomes containing virion envelope components adsorbed via protein moieties. Although pinocytosis may be present, the evidence strongly suggests that the observed adsorption is not mediated via this mechanism. Differential binding of liposomes with different protein compositions and the ability to remove bound label with trypsin offer proof that the adsorption is specific and independent of internalization.

Correspondence of liposome binding site with virion binding site was demonstrated by a number of criteria. The liposomes were capable of inhibiting virion binding if either VE1 or VE2 was present. The opposite situation, virions inhibiting liposome adsorption, was also observed. These results suggest that the binding sites for the VE1/VE2 liposomes and virions are either identical or closely related. The enzyme susceptibility profiles for both liposome and virus receptor were identical. These data are supportive of our previous data which suggested that the virus receptor is proteinaceous in nature (20).

In a number of preliminary experiments (data not shown), the binding moieties of viable virions were indirectly examined. Antisera which recognized a number of the four major envelope components were assayed for their effect on virus binding. After removal of immune complexes from the pooled human sera (12, 13), the residual sera neutralized both infectivity and binding. Monospecific and monoclonal sera directed against either VE1 or VE2 also negated virion binding. As viral binding is the first step in infection, in vitro neutralization of virions by these sera is accomplished at this stage. Whether this mechanism is responsible for neutralization in vivo remains to be seen, though evidence has been presented suggesting that VE1 and VE2 are the major neutralizing targets in vivo (D. Thorley-Lawson, submitted for publication).

VE1 or VE2 is capable of inhibiting virion binding, but both fail to bind stably unless in the presence of the other. The interfering liposomes, with either component, are replaced by stably bound virions; as such, there is only a weak affinity between the receptor and the individual glycoproteins. This situation suggests two alternative molecular models for binding. The first has VE1 and VE2 recognizing separate but associated binding cavities in which the binding of one protein increases the affinity of the second cavity, analogous to cooperative binding sites. Therefore, only when both components are present is the binding stable. This model is unlikely since a mixture of VE1-containing liposomes and those with VE2 does not demonstrate

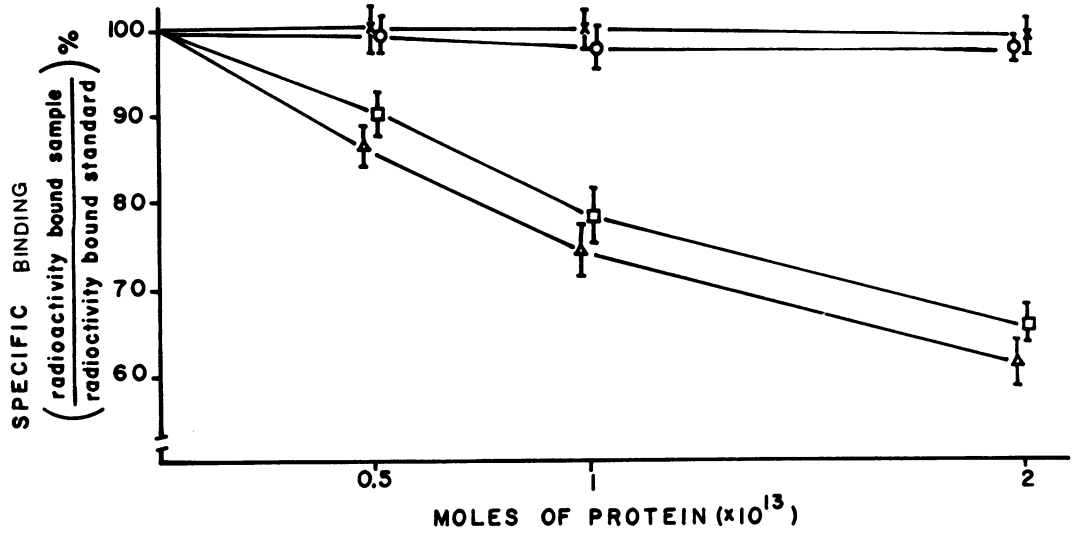

FIG. 6. Inhibition of EB virion binding by virion envelope components reconstituted in liposomes. ^{125}I-labeled virions were incubated for 20 min with 4×10^6 Raji cells while in the presence of various amounts of liposomes containing the different virion envelope components. The binding assay was performed as described in Materials and Methods. Protein determinations were performed by the method of Lowry et al. (22). Symbols: □, VE1; △, VE2; ×, VE3; ○, VE5. The results represent the average of six repetitions with two different liposome batches (± standard deviation). Similar results were obtained with Daudi cells. Binding of virus to the negative controls was unchanged in the presence of liposomes at 4% of gross counts; this level was utilized to calculate 100% specific inhibition.

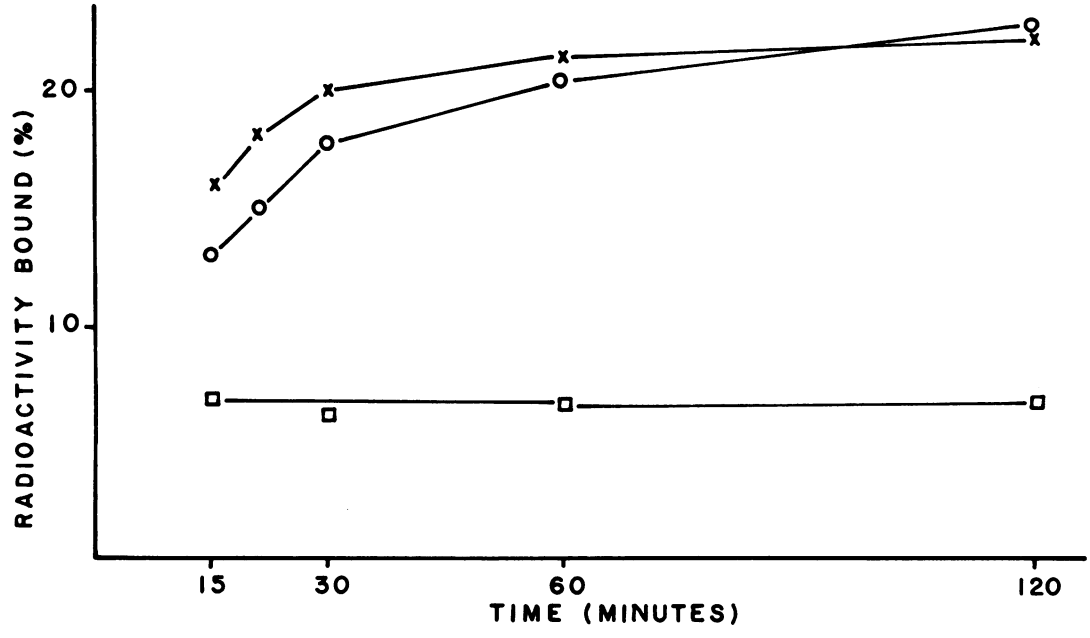

FIG. 7. Binding of EB virions in the presence of VE2. ^{125}I-labeled EB virions were coincubated with 10^{-13} mol of VE2 reconstituted in liposomes and 4×10^6 Raji cells. In parallel, cells and virions were incubated in the absence of liposomes. At various time points samples were assayed for virus bound. Symbols: ×, Raji cells in the absence of liposomes; ○, Raji cells in the presence of liposomes; □, negative controls, which acted identically in the presence or absence of liposomes. The results shown are the averages of three trials. A similar profile was obtained with VE1 liposomes.

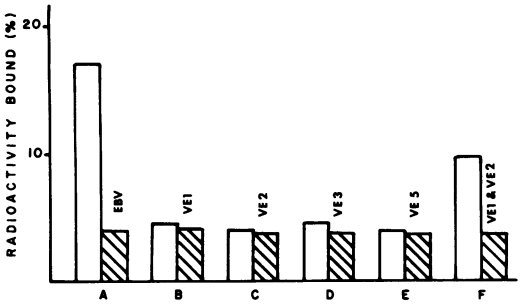

FIG. 8. Binding of ^{125}I-labeled virion envelope components to receptor-positive and -negative target cells. Liposomes containing iodinated virion envelope components were incubated with Raji (open bars) or YAC (slashed bars) cells as described in Materials and Methods. (A) Binding of the original virion from which the individual components were isolated, (B) liposomes containing VE1, (C) VE2 liposomes, (D) VE3 liposomes, (E) VE5 liposomes, and (F) liposomes containing both VE1 and VE2. The results are from four independent assays.

specific adsorption. Additionally, according to this model the presence of one component would accelerate virion binding by "activating" the opposite cavity, a situation which was not observed. The preferred model is one in which the two components act in conjunction as a single binding component. In this model, either molecule would recognize its part of the cavity but be incapable of establishing a stable bond. It could, however, interfere competitively with other entities vying for the receptor. It is beyond

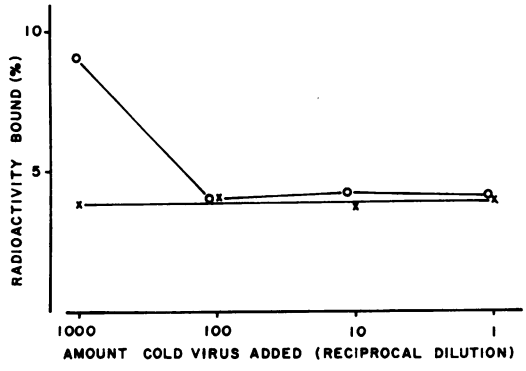

FIG. 9. Competitive inhibition of iodinated VE1/VE2 liposomes by nonlabeled virions. Various dilutions of purified viable virions in RPMI 1640 medium and 10% total calf serum were coincubated with VE1/VE2 liposomes and Raji (O) or K562 (×) cells. The binding assay was performed as described. The initial dilution of virion represented approximately 10^{-12} mol of virus. Similar results were obtained with Daudi cells.

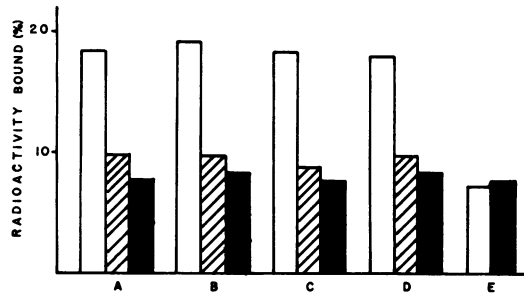

FIG. 10. Failure of additional virion envelope components to enhance the level of binding. Liposomes were reconstituted around thymidine-labeled nucleocapsids and nonlabeled envelope components. These were then incubated with cells for 2 h at 37°C. After the incubation and washing, a sample of Raji cells (slashed bar) was treated with an equal volume of 0.25% trypsin (Sigma Chemical Co., St. Louis, Mo.; product T2884) and washed again. Raji cells treated with balanced salt solution were run in parallel (open bar) as were negative controls (black bar). (A) Liposomes with VE1 and VE2; (B) VE1, VE2, and VE3 liposomes; (C) VE1, VE2, and VE5-containing liposomes; (D) liposomes formed in the presence of all four components; and (E) liposomes formed in the absence of any envelope components. Similar results were obtained with Daudi cells.

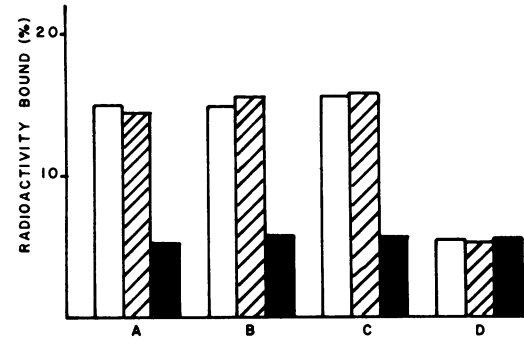

FIG. 11. Enzyme treatment of target cells prior to binding of liposomes and virus. Target cells were trypsinized or neuraminidase treated as follows: 4×10^6 cells were incubated in 0.125% trypsin (black bar) (Sigma; product T2884) for 5 min at room temperature or 25 U of *Vibrio cholerae* neuraminidase (slashed bar) (BDH, Poole, England; product 39027) for 30 min at 37°C in balanced salt solution. The treated cells were then washed twice and tested for binding of liposomes containing thymidine-labeled nucleocapsids and nonlabeled virion envelope components. Nontreated cells were incubated in parallel in balanced salt solution alone (open bar). (A) Raji cells and virions (adjusted to the binding levels of the virion envelope liposomes), as previously reported (20), (B) Raji cells and liposomes containing VE1 and VE2, (C) Raji cells and liposomes containing all four components, (D) negative control cells (K562) with liposomes containing the four components. The averages of three assays are shown.

the scope of this study to present conclusive evidence for one of these models.

In summary, it has been shown that EB virus binding is mediated by only two of the virion envelope components, VE1 and VE2. For stable binding to occur, both components must be present. Previously, we reported an EB virus receptor which selectively binds only one of the two virus substrains, the P3HR-1–derived virus (36). As the only demonstrated structural difference between the two viral substrains is the ratio of VE1 to VE2 (6; unpublished data), these findings are consistent with the idea that a complex of the two components is the basis for the virus' extreme target selectivity.

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