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# A Monoclonal Antibody to Glycoprotein gp85 Inhibits Fusion but Not Attachment of Epstein-Barr Virus

NANCIMAE MILLER AND LINDSEY M. HUTT-FLETCHER*

*Departments of Comparative and Experimental Pathology and Immunology and Medical Microbiology, University of Florida, Gainesville, Florida 32610*

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**Epstein-Barr virus (EBV) codes for at least three glycoproteins, gp350, gp220, and gp85. The two largest glycoproteins are thought to be involved in the attachment of the virus to its receptor on B cells, but despite the fact that gp85 induces neutralizing antibody, no function has been attributed to it. As an indirect approach to understanding the role of gp85 in the initiation of infection, we determined the point at which a neutralizing, monoclonal antibody that reacted with the glycoprotein interfered with virus replication. The antibody had no effect on virus binding. To examine the effect of the antibody on later stages of infection, the fusion assay of Hoekstra and colleagues (D. Hoekstra, T. de Boer, K. Klappe, and J. Wilshaut, Biochemistry 23:5675–5681, 1984) was adapted for use with EBV. The virus was labeled with a fluorescent amphiphile that was self-quenched at the high concentration obtained in the virus membrane. When the virus and cell membrane fused, there was a measurable relief of self-quenching that could be monitored kinetically. Labeling had no effect on virus binding or infectivity. The assay could be used to monitor virus fusion with lymphoblastoid lines or normal B cells, and its validity was confirmed by the use of fixed cells and the Molt 4 cell line, which binds but does not internalize the virus. The monoclonal antibody to gp85 that neutralized virus infectivity, but not a second nonneutralizing antibody to the same molecule, inhibited the relief of self-quenching in a dose-dependent manner. This finding suggests that gp85 may play an active role in the fusion of EBV with B-cell membranes.**

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that can cause infectious mononucleosis (13) and diffuse lymphoproliferative disorders (6); it is also associated with the development of B-cell neoplasia and nasopharyngeal carcinoma. Although the virus binds to and infects both epithelial cells and B lymphocytes (35), difficulties in establishing suitable epithelial cell cultures in vitro have meant that its replication has been studied almost exclusively in lymphocytes. The infection of lymphocytes is initiated by the attachment of EBV to a 145-kilodalton cell membrane glycoprotein, CR2, which also serves as the receptor for the complement C3d fragment (9, 28). The binding of EBV to CR2 is mediated by the attachment of at least one virus membrane glycoprotein, gp350 (27, 37), and possibly also by the attachment of a second glycoprotein, gp220 (40). Glycoproteins gp350 and gp220 are encoded by the same open reading frame from which an intron is removed, without change in reading frame, to produce gp220 (3), and monoclonal antibodies that recognize gp350 and gp220 are capable of inhibiting virus binding (27). The envelope of EBV contains, however, at least one additional less abundant glycoprotein, gp85. Although no function has yet been ascribed to this molecule, antibodies to it can neutralize virus infectivity (36), implying that it may play some role in the initiation of cell infection. As an indirect approach to understanding the function of gp85, we set out to determine the point at which a monoclonal antibody to the glycoprotein, which neutralizes virus infectivity, interferes with the replication cycle.

Assays for virus attachment are well established, but information about the penetration of virus comes principally from microscopy studies which are extremely painstaking and difficult to quantitate. The information that is available from these studies suggests that the binding of virus to lymphoblastoid cells is followed by the fusion of the virus envelope with the plasmalemma at the cell surface (25, 34). To analyze EBV penetration, we therefore adapted an assay that was developed by Hoekstra and colleagues (15) to study Sendai virus fusion. The assay makes use of a fluorescent amphiphile, octadecyl rhodamine B chloride ($R_{18}$), which can be readily inserted into biologic membranes. At high concentrations, such as can be achieved in virus membranes, the probe is self-quenching; at the lower concentrations that result from the fusion of virus and cell membranes, there is measurable relief of self-quenching and an increase in fluorescence that can be monitored kinetically. We report here that this assay can be used to monitor the fusion of EBV with lymphoblastoid cell lines or normal peripheral B cells, which are reported to internalize the virus by endocytosis (25, 38), and to demonstrate that a neutralizing antibody specific for gp85 fails to inhibit virus attachment but can interfere with fusion.

## MATERIALS AND METHODS

**Lymphoblastoid cell lines.** Cell lines were grown at 37°C and diluted at least biweekly in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal calf serum, 100 IU of penicillin per ml, and 100 μg of streptomycin per ml. These lines were Raji (31), an EBV genome-positive, nonproducing human B-cell line that expresses CR2; Daudi (20), an EBV genome-positive, nonproducing human B-cell line that currently, in our laboratory, does not express CR2; Molt 4 (24), an EBV genome-negative human T-cell line that expresses CR2 but cannot internalize the virus (23); P3HR1-Cl13 (14), a superinducible EBV-producing human B-cell line; MCUV5, an EBV-producing marmoset cell line; and P3HR1-C15 (14), an EBV genome-positive human B-cell line derived from the same

* Corresponding author.

parent line as P3HR1-Cl13, which currently, in our laboratory, does not produce virus and does not express CR2. The last three lines were gifts from George Miller, Yale University, New Haven, Conn.

**Antibodies.** Four antiviral hybridoma antibodies were used: E8D2 (2), an immunoglobulin G2a antibody that recognizes the EBV-induced early membrane protein p105; F-2-1 (36), an immunoglobulin G2a antibody that recognizes gp85; 72A1 (17), an immunoglobulin G1 antibody that recognizes gp350 and gp220; and E1D1, an immunoglobulin G1 antibody that also recognizes gp85 and was made in our laboratory as previously described (1). All four antibodies were purified from culture supernatants by chromatography on protein A agarose. In addition to these antiviral antibodies, two monoclonal antibodies that react with CR2 were used: OKB7, which blocks virus binding, and HB5, which does not block receptor-binding sites (26). These antibodies were gifts from Barry Myones, University of North Carolina at Chapel Hill.

**Virus production and radiolabeling.** Virus-producing cells were induced with 30 ng of 12-*O*-tetradecanoylphorbol-13-acetate per ml, and after 7 days, virus was collected from the spent culture medium. The cells were centrifuged at 4,000 × *g* for 10 min to remove cells; 100 μg of bacitracin per ml was added to the clarified supernatant, and the virus was pelleted by centrifugation at 20,000 × *g* for 90 min. Pellets were suspended in 1/250 of the original volume of medium containing 100 μg of bacitracin per ml, reclarified by centrifugation three to four times at 400 × *g*, and filtered through a 0.45-μm-pore filter (Acrodisc; Gelman Sciences, Inc., Ann Arbor, Mich.). P3HR1-Cl13 virus was intrinsically labeled with [$^3$H]thymidine (Amersham Corp., Arlington Heights, Ill.) by feeding cells with medium containing 100 μM hypoxanthine and 0.4 μM aminopterin, inducing them when they reached maximum density (day 0) with 30 ng of 12-*O*-tetradecanoylphorbol-13-acetate per ml in the presence of 2 μCi of [$^3$H]thymidine (specific activity, 5 Ci/mmol) per ml and an additional 2 μCi of [$^3$H]thymidine (specific activity, 52 Ci/mmole) per ml on day 3, and harvesting on day 7. All virus stocks were stored at −70°C.

**Incorporation of $R_{18}$ into virus membranes.** A stock solution of 13 nmol of $R_{18}$ (Molecular Probes, Inc., Junction City, Oreg.) per μl was prepared in chloroform-methanol (1:1) and stored at −20°C. The probe was incorporated into virus membranes by a modification of the method of Hoekstra and colleagues (15). Approximately 5 ml of virus was sieved over Bio-Gel A-150m (Bio-Rad Laboratories, Richmond, Calif.), pelleted by centrifugation, and suspended in 2 ml of phosphate-buffered saline (pH 7.2). The stock probe (3 μl) was dried under nitrogen and solubilized in ethanol, and 15 μl of this solution containing 15 nmol of $R_{18}$ was added to 500 μl of sieved virus with vortexing. The probe and virus were incubated at room temperature in the dark for 1 h, after which the virus and unincorporated $R_{18}$ were separated by chromatography on Sephadex G-75 (Sigma Chemical Co., St. Louis, Mo.). Labeled virus was stored at −70°C.

**Fluorescence measurement.** $R_{18}$-labeled virus (50 to 100 μl) was added to pellets of $2 \times 10^6$ cells and incubated for 1 h at 4°C on ice in the dark. Cells were washed four times with ice-cold phosphate-buffered saline, suspended in 400 μl, and transferred (time zero) to the microcuvette of an Aminco Bowman spectrophotofluorometer equipped with a chart recorder for continuous monitoring of fluorescence (excitation and emission wavelengths of 560 and 585 nm, respectively, for the incorporated probe). The cuvette was equipped with a magnetic stirrer and was kept in a warming chamber at 37°C. At the end of the assay, Triton X-100 (1% [vol/vol] final concentration) was added to allow the measurement of fluorescence that would be obtained upon infinite dilution of the fluorophore. Fluorescence was expressed in arbitrary units (a.u.). For many experiments, the fluorescence scale was calibrated such that the residual fluorescence of membranes at time zero was taken as the zero level and the value obtained after the addition of Triton X-100, corrected for sample dilution, was taken as 100% (infinite dilution).

**Virus titration and neutralization.** The infectivity of the virus was measured in terms of its ability to induce human peripheral B lymphocytes to secrete immunoglobulin, as previously described (18). Briefly, leukocytes were obtained from heparinized blood by flotation on Ficoll-Hypaque and depleted of T cells by a double cycle of rosetting with 2-aminoethylisothiouronium bromide-treated sheep erythrocytes (30) and centrifugation over 60% Percoll (Pharmacia Fine Chemicals, Piscataway, N.J.). A total of 200,000 cells were incubated with or without virus in 100 μl of medium in 96-well round-bottomed tissue culture plates. After 6 days in culture, 100 μl of medium was added to each well. On day 12, the immunoglobulin concentrations in the media were measured by a double-sandwich enzyme-linked immunosorbent microassay with the appropriate concentrations of rabbit anti-human immunoglobulin, peroxidase-conjugated rabbit anti-human immunoglobulin, and the substrate hydrogen peroxide with 5-aminosalicylic acid. The colorimetric change was measured at 492 nm. The ability of the antibody to neutralize infectivity was determined by preincubating the virus for 1 h at room temperature with an equal volume of normal rabbit antibody at 100 μg per ml or with mixtures of rabbit antibody and test antibody with concentrations adjusted so that the total amount of immunoglobulin remained constant at 100 μg per ml. All antibodies were heated for 30 min at 56°C to inactivate complement.

**Virus binding assays.** The ability of radiolabeled virus to bind specifically to CR2 was determined by the use of receptor-positive and -negative cells that had been briefly fixed with ice-cold 0.1% paraformaldehyde. The virus was incubated with fixed cells for 60 min at 4°C, cells were washed five times, and the acid-precipitable radioactivity remaining attached to cells was counted. The ability of antibody to interfere with virus binding was determined by preincubating the virus with antibody for 1 h at room temperature.

## RESULTS

**Effect of antibodies on virus infectivity and binding.** Of the four monoclonal antibodies used in this work, two, 72A1 and F-2-1, both of which have previously been reported to neutralize virus infectivity in the absence of complement (17, 36), inhibited the ability of the transforming strain of virus, MCUV5, to induce immunoglobulin synthesis; less of antibody 72A1 was required for this purpose (Table 1). A comparison of the ability of the two neutralizing antibodies and one of the nonneutralizing antibodies to inhibit virus binding indicated that, as expected, the nonneutralizing antibody E8D2 had no inhibitory effect and, at high concentrations, even slightly increased the amount of bound radioactivity, perhaps because it mediated the attachment of virus to Fc receptors as well as permitting binding to CR2 (Table 2). In contrast, the ability of 72A1 to neutralize infectivity could be accounted for by its ability to block virus binding. Most interesting, however, was the observation that anti-

TABLE 1. Effect of monoclonal antibodies on ability of MCUV5 virus to induce immunoglobulin synthesis by fresh T-cell-depleted human leukocytes

| Monoclonal antibody | Monoclonal antibody:normal rabbit antibody ratio (ng per culture) | Immunoglobulin concn (ng/ml) with: | | % Inhibition |
|---|---|---|---|---|
| | | Antibody alone | Antibody + virus | |
| F-2-1 | 1,000:0 | 426 | 5,958 | 62 |
| | 300:700 | 256 | 9,396 | 41 |
| | 100:900 | 268 | 15,628 | 1 |
| | 30:970 | 447 | 17,862 | 0 |
| | 10:990 | 329 | 17,696 | 0 |
| E1D1 | 1,000:0 | 151 | 14,998 | 5 |
| | 300:700 | 120 | 15,963 | 0 |
| | 100:900 | 224 | 17,583 | 0 |
| | 30:970 | 132 | 17,887 | 0 |
| | 10:990 | 144 | 15,656 | 1 |
| 72A1 | 1,000:0 | 208 | 686 | 96 |
| | 300:700 | 318 | 2,429 | 85 |
| | 100:900 | 404 | 7,604 | 52 |
| | 30:970 | 232 | 10,303 | 35 |
| | 10:990 | 325 | 16,632 | 0 |
| E8D2 | 1,000:0 | 199 | 15,250 | 4 |
| | 300:700 | 316 | 14,821 | 6 |
| | 100:900 | 352 | 16,487 | 0 |
| | 30:970 | 124 | 17,775 | 0 |
| | 10:990 | 140 | 17,379 | 0 |
| None | 0:1,000 | 263 | 15,840 | |

body F-2-1, despite its ability to neutralize infectivity, resembled the nonneutralizing antibodies in its effects on virus binding. Although more of F-2-1 than of the 72A1 antibody was required to neutralize infectivity, even the increase of F-2-1 concentrations by the same order of magnitude required to equal neutralization by 72A1 failed to block virus attachment. A fusion assay was therefore established to determine whether antibody F-2-1 inhibited infectivity at this subsequent step in replication.

**Effect of $R_{18}$ on the attachment of virus.** In adapting the fluorescence assay of Hoekstra and colleagues for use with EBV, our first concern was to ensure that the labeling of virus with the fluorescent probe did not qualitatively or quantitatively affect virus binding. We therefore labeled the virus metabolically with [$^3$H]thymidine, divided the labeled virus into three aliquots, left one aliquot untreated, labeled another with an ethanolic solution of $R_{18}$, and mock labeled the third aliquot with ethanol containing no $R_{18}$. Both labeled and mock-labeled virus preparations were chromatographed on Sephadex G-75. Approximately half the bindable virus was lost during the labeling and mock-labeling procedures; however, if the amount of radioactivity bound was expressed as a percentage of that added, it could be seen that labeling had no effect on the ability of virus to bind to receptor-positive cells (Table 3). There was also no increase in nonspecific binding to the receptor-negative P3HR1-C15 line. We further confirmed the specificity of binding by demonstrating that (i) preincubation of virus with antibody 72A1 inhibited the ability of virus to bind, (ii) preincubation of cells with the anti-CR2 antibody, OKB7, which normally blocks virus binding (26), inhibited labeled-virus binding, and (iii) preincubation with HB5, a monoclonal antibody to CR2 that does not block the binding sites of CR2, appropriately failed to inhibit the binding of labeled virus (Table 4).

TABLE 2. Effect of monoclonal antibodies on ability of $^3$H-labeled EBV to bind to receptor-positive cells[a]

| Antibody concn (μg/ml) | Total acid-precipitable radioactivity (cpm) bound in the presence of monoclonal antibody | | |
|---|---|---|---|
| | 72A1 | F-2-1 | E8D2 |
| 600 | 177 | 5,831 | 4,891 |
| 400 | 162 | 5,163 | 5,080 |
| 200 | 187 | 5,899 | 5,832 |
| 100 | 220 | 5,044 | 5,379 |
| 50 | 417 | 5,549 | 5,682 |
| 25 | 1,183 | 5,038 | 5,297 |
| 12.5 | 2,296 | 4,760 | 5,494 |

[a] Counts bound to receptor-negative cells, 110; counts bound in the absence of antibody, 4,764.

TABLE 3. Effect of $R_{18}$ labeling on ability of $^3$H-labeled EBV to bind to receptor-positive and -negative cells

| Virus treatment | Virus dilution | Total acid-precipitable radioactivity (cpm) bound to: | | % Acid-precipitable radio activity[a] bound to: | |
|---|---|---|---|---|---|
| | | Raji[b] | P3HR1-Cl5[c] | Raji | P3HR1-Cl5 |
| None | Neat | 13,284 | 543 | 28.9 | 1.2 |
| | 1/2 | 6,346 | —[d] | 27.6 | — |
| | 1/4 | 2,704 | 217 | 23.5 | 1.8 |
| | 1/8 | 1,408 | — | 24.5 | — |
| Mock labeled | Neat | 5,816 | 113 | 29.4 | 0.6 |
| | 1/2 | 2,663 | — | 26.8 | — |
| | 1/4 | 1,366 | 38 | 27.5 | 0.7 |
| | 1/8 | 758 | — | 30.6 | — |
| Labeled with $R_{18}$ | Neat | 6,962 | 248 | 25.5 | 1.0 |
| | 1/2 | 3,707 | — | 27.1 | — |
| | 1/4 | 1,831 | 104 | 26.8 | 1.5 |
| | 1/8 | 1,016 | — | 29.7 | — |

[a] Percent acid-precipitable radioactivity = 100 × radioactivity bound/radioactivity added.
[b] Raji, Receptor-positive cells.
[c] P3HR1-Cl5, Receptor-negative cells.
[d] —, Not done.

**Effect of $R_{18}$ on infectivity of virus.** Although the incorporation of $R_{18}$ into the virus membrane had no apparent effect on virus attachment, it remained possible that the probe interfered with some subsequent event in virus replication. To examine this possibility, a comparison was made of the ability of labeled and mock-labeled MCUV5 virus to induce immunoglobulin synthesis in cultures of T-cell-depleted pe-

TABLE 4. Effect of monoclonal anti-EBV and anti-CR2 antibodies on ability of $R_{18}$-labeled and $^3$H-labeled EBV to bind to receptor-positive cells

| Antibody (amt [μg]) | Amt of radioactivity (cpm) remaining with: | | | |
|---|---|---|---|---|
| | $R_{18}$-labeled virus | | Mock-labeled virus | |
| | Total | % | Total | % |
| None | 1,904 | 23.5 | 2,789 | 23.9 |
| 72A1 (10) | 123 | 1.5 | 231 | 1.9 |
| OKB7 (5) | 198 | 2.4 | 180 | 1.5 |
| HB5 (5) | 1,390 | 17.1 | 2,308 | 19.8 |

TABLE 5. Effect of $R_{18}$ labeling on ability of MCUV5 virus to induce immunoglobulin synthesis by fresh T-cell-depleted human leukocytes

| Virus dilution | Immunoglobulin concn (ng/ml) with[a]: | |
|---|---|---|
| | $R_{18}$-labeled virus | Mock-labeled virus |
| 1/5 | 24,754 | 22,366 |
| 1/10 | 45,720 | 23,836 |
| 1/20 | 38,609 | 23,639 |
| 1/40 | 39,902 | 27,404 |
| 1/80 | 42,326 | 30,505 |
| 1/160 | 33,921 | 21,093 |
| 1/320 | 18,168 | 15,999 |
| 1/640 | 3,970 | Not done |

[a] Immunoglobulin concentration in the absence of virus, 1,138.

ripheral leukocytes (Table 5). There was no indication that the incorporation of probe into the virus had any effect on its biologic activity.

**Changes in fluorescence after interaction of $R_{18}$-labeled virus with lymphoblastoid cells.** We first measured the changes in fluorescence emission that occurred as virus bound to Raji cells was warmed in the cuvette of the spectrophotofluorometer (Fig. 1). Fluorescence increased gradually over approximately 28 to 30 min, after which time little further change was detectable in this and subsequent experiments. Approximately 55% of the maximal fluorescence at infinite dilution (determined by the addition of Triton X-100) was reached; this value was reproducible for any one batch of virus but varied slightly between batches. The maximal value obtained with any batch of virus was 75%. Parallel analysis of the receptor-negative Daudi line confirmed that $R_{18}$-labeled virus failed to bind to these cells and showed that there was no significant diffusion of residual free or incorporated probe from virus preparations into cell membranes during 1 h of incubation at 4°C.

Further indication that the relief of self-quenching was measuring a membrane fusion event was obtained by using fixed Raji cells and the Molt 4 cell line (Fig. 2). The increase in fluorescence emission that occurred when virus was bound to Raji cells and warmed to 37°C was almost completely eliminated when the cells were prefixed with paraformaldehyde. When Molt 4 cells were substituted for Raji cells, there was a slight reduction in the amount of virus that was bound, a result in agreement with our experience that Molt 4 cells express fewer CR2 receptors than Raji cells do (data not shown). However, there was no detectable relief of self-quenching of the bound probe, a result compatible with the reported inability of the virus to fuse with Molt 4 cell membranes.

**Changes in fluorescence after interaction of $R_{18}$-labeled virus with normal B cells.** Two independent studies have demonstrated that although EBV fuses with the plasmalemma of lymphoblastoid cells, it is internalized in normal B cells by endocytosis before any fusion of cell and virus membrane occurs (25, 38). However, if fusion was subsequently to occur within an endocytic vesicle, it seemed likely that the event might still be detectable by the use of $R_{18}$-labeled virus. We confirmed this possibility by measuring the relief of self-quenching of $R_{18}$-labeled MCUV4 virus bound to T-cell-depleted peripheral leukocytes (Fig. 3). Although considerably less virus bound to normal B cells on a per cell basis and a smaller spontaneous increase in fluorescence was achieved with normal B cells than with lymphoblastoid cells, a measurable signal was obtained. In this experiment, the maximum increase in fluorescence was approximately 55%, although with many batches of virus, the value was slightly lower.

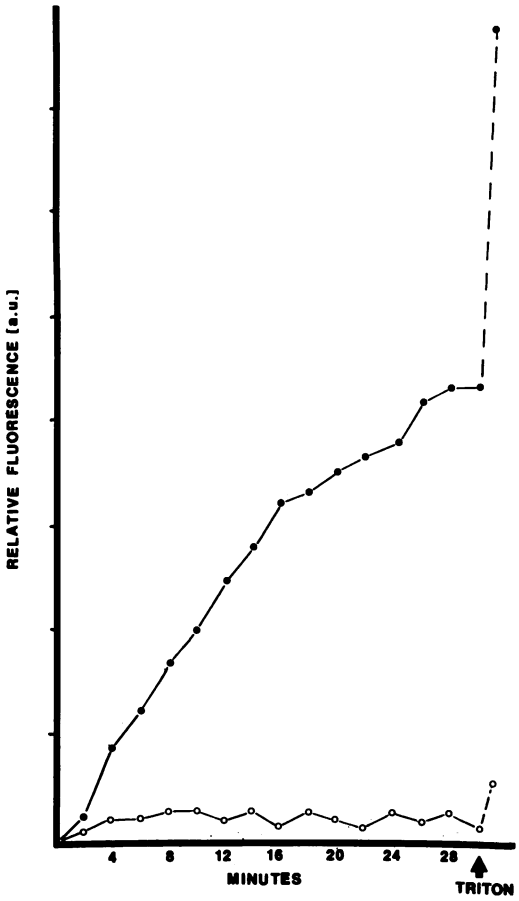


FIG. 1. Relief of self-quenching of $R_{18}$-labeled virus bound to receptor-positive Raji cells (●——●) and receptor-negative Daudi cells (○——○). The point at which Triton X-100 was added to measure maximum relief of self-quenching of bound probe within the volume of the cuvette (infinite dilution) is indicated by the arrow. Changes in the relief of self-quenching after addition of Triton X-100 of probe bound to Raji cells (●- - -●) or Daudi cells (○- - -○) are shown.

**Effect of antibodies on relief of self-quenching of incorporated probe.** Preincubation of P3HR1-Cl13 virus with the nonneutralizing antibody, E8D2, had no effect on the amount of $R_{18}$-labeled virus that bound to Raji cells or on the relief of self-quenching of the incorporated probe after the cells were warmed to 37°C (Fig. 4). In contrast, although preincubation of virus with antibody F-2-1 did not affect the amount of fluorophore that bound, it effectively inhibited the relief of self-quenching. This result suggested that the neutralization of virus infectivity by F-2-1 might occur because the antibody interferes with the fusion of virus with the cell membrane. To explore this hypothesis further and to rule out the possibility that the effects of the antibody were unique to the P3HR1-Cl13 virus, we repeated the experiment with $R_{18}$-labeled MCUV5 virus and also included an additional antibody, E1D1, which, despite being a subclass of antibody different from either E8D2 or F-2-1, provided an additional control as a nonneutralizing antibody that recognizes gp85. Of the three antibodies, only F-2-1 influenced the relief of self-quenching, and the inhibition was dose dependent

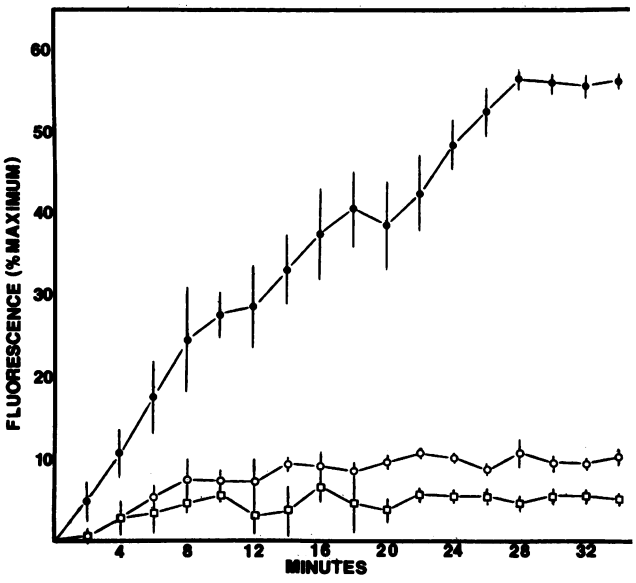


FIG. 2. Comparison of relief of self-quenching of P3HR1-Cl13 virus bound to Raji cells (●), fixed Raji cells (○) or Molt 4 cells (□). The increase in fluorescence is expressed as a percentage of the maximum release obtained with each cell line after the addition of Triton X-100 (infinite dilution). The average maximum fluorescence values for the cell lines were as follows: Raji, 100 a.u.; fixed Raji, 97 a.u.; Molt 4, 75 a.u. Vertical lines indicate the standard deviations of the means of three experiments with the same batch of labeled virus.

(Table 6). Sixty percent inhibition was achieved with approximately 3 μg of antibody, in reasonable experimental agreement with the amount of antibody necessary to inhibit virus induction of immunoglobulin synthesis by the same percentage.

## DISCUSSION

Previous studies of the functions of EBV membrane glycoproteins have concentrated on the largest and most abundant molecules, gp350 and gp220. These molecules play crucial roles as virus attachment proteins, and a majority of the neutralizing activity in normal sera can be accounted for by antibody that reacts with them (39). However, the predominant anti-membrane activity in neutralizing sera taken from patients during the acute phase of infectious mononucleosis is directed against gp85 (32). This observation, together with the fact that monoclonal antibodies to gp85 can neutralize virus infectivity, indicates that the smaller and less abundant viral glycoprotein gp85 is also important to virus replication. The experiments described in this paper suggest that gp85 is involved in the fusion of virus with the cell membrane.

The fluorescent amphiphile $R_{18}$ has been used by several workers to study the interaction of viruses with intact cells and liposomes (4, 15, 16, 21), and fluorescence dequenching has been inferred to reflect a process of virus-membrane

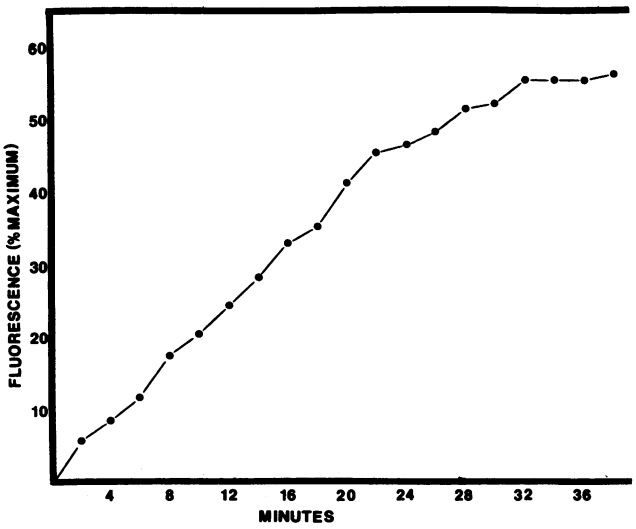


FIG. 3. Relief of self-quenching of $R_{18}$-labeled MCUV5 virus bound to fresh T-cell-depleted leukocytes expressed as a percentage of the maximum release obtained after the addition of Triton X-100 (infinite dilution).

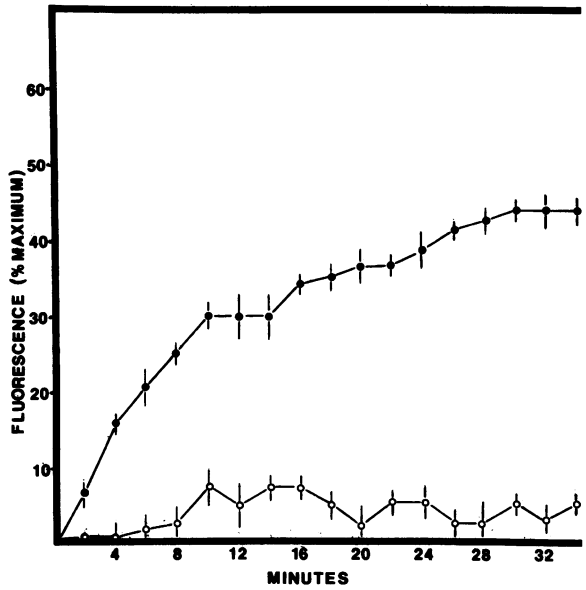


FIG. 4. Effect of preincubation with 100 μg of monoclonal antibodies on relief of self-quenching of $R_{18}$-labeled P3HR1-Cl13 virus added to Raji cells. ●, Preincubation with antibody E8D2; ○, preincubation with antibody F-2-1. Vertical lines indicate the standard deviations of the means of three experiments with the same batch of labeled virus. The average maximum fluorescence values obtained after the addition of Triton X-100 were 72 a.u. for virus preincubated with E8D2 and 76 a.u. for virus preincubated with F-2-1.

TABLE 6. Effect of antibody on relief of self-quenching of $R_{18}$-labeled virus added to T-cell-depleted human leukocytes

| Antibody | Amt added (μg) | Fluorescence (% of maximum) |
|---|---|---|
| F-2-1 | 100 | 6.1 |
| | 50 | 4.7 |
| | 25 | 5.6 |
| | 12.5 | 6.1 |
| | 6.25 | 9.4 |
| | 3.13 | 13.3 |
| | 1.56 | 24.3 |
| E8D2 | 100 | 33.1 |
| E1D1 | 100 | 29.7 |
| None | 0 | 31.1 |

fusion. The behavior of $R_{18}$-labeled EBV, the relief of self-quenching of virus bound to Raji cells, and the failure of virus bound to either fixed Raji cells or Molt 4 cells provide compelling corroborative support for this conclusion. Fixed cells are resistant to virus-membrane fusion (21), and Molt 4 cells are reported to bind but not internalize virus (23). The labeling procedure had no apparent effect on the specificity of binding or the amount of EBV that bound to lymphoblastoid cells. This result is in agreement with the effect of labeling on the attachment of Sendai virus (16), although labeling of vesicular stomatitis virus enhances binding by twofold, possibly because the probe is positively charged and increases the net charge of the virus (4). Labeled virus also retained its infectivity, as judged by its ability to induce immunoglobulin synthesis by cultured T-cell-depleted peripheral leukocytes.

The kinetics of fusion with Raji cells and normal lymphocytes were very similar, with a slight but measurable changes occurring within 2 min of warming the virus-cell complexes in the cuvette of the spectrophotofluorometer. This observation was a little suprising because fusion is reported to occur at the plasmalemma of lymphoblastoid cells (25, 34) and virus is reported to enter normal B cells by endocytosis (25, 38). A 1- to 2-min lag, corresponding to the time required for the initial entry of ligands, toxins, and virions into an acidic compartment after receptor-mediated endocytosis (5), has been reported for the relief of self-quenching of $R_{18}$-labeled vesicular stomatitis virus bound to Vero cells (4). However, since EBV appears capable of fusing with the plasma membrane at the surface of the cell or after endocytosis, this observation may simply mean either that the virus can enter normal B cells by both routes or that fusion with an endocytic vesicle wall occurs very rapidly after uptake, even before the virus is exposed to a low pH. Experiments using inhibitors of endocytosis, pH-sensitive fluorescent probes, and lysosomotropic agents may help answer this question and perhaps determine whether one or both pathways are relevant to productive infection.

The maximal percentage of increase in fluorescence with lymphoblastoid cells was 75%. This value was never achieved with normal B cells, and the relief of self-quenching of virus bound to normal B cells was frequently slightly less than that obtained with Raji cells. It is possible that this result reflects a higher degree of nonspecific binding to normal B cells, that there is a reduced capacity to measure fluorescence intensity inside rather than outside a cell, or that there is a diminished ability of the probe to diffuse within the confines of an endocytic vesicle membrane and relieve self-quenching. The leukocytes used for the experiments described in this paper were not depleted of mononuclear cells; phagocytosis by this cell type was not, however, responsible for the increase in fluorescence observed in leukocyte cultures. Depletion of monocytes from approximately 50% to approximately 10% had no effect on the magnitude of the response (data not shown), and addition of antibody, which might be expected to increase rather than decrease uptake by phagocytic cells, did not enhance the relief of self-quenching. Even if EBV is phagocytosed into mononuclear cells, the virus taken up by this route may not fuse with endocytic vesicle membranes.

Elegant studies with purified recombinant gp350 and gp220 have implicated these two proteins in the internalization process (37). Beads coated with the recombinant proteins not only bound to the EBV receptor on normal B cells but were also internalized by endocytosis; 24 h later, 10% of the beads were discharged free into the cytoplasm. However, the inhibition of the relief of self-quenching of $R_{18}$-labeled virus by a neutralizing antibody to gp85 that does not affect virus binding and the failure of a nonneutralizing antibody to the same molecule to influence fluorescence increase are powerful, if indirect, evidence for the involvement of gp85 in the fusion of virus with the cell membrane, possibly leading to penetration. It remains possible, though perhaps unlikely in view of the fact that gp85 is a minor protein component of the viral membrane (12, 29), that antibody F-2-1 inhibits some conformational change in gp350 or gp220 that is important for penetration. It is also conceivable, however, that the discharge of gp350- and gp-220-coated beads into the cytoplasm of B cells is not completely representative of a normal infectious process; at least three glycoproteins of herpes simplex virus, gB, gD, and gH, have been implicated in penetration (10, 11, 33). The gene encoding gp85 has recently been mapped to the BXLF2 open reading frame of EBV DNA (12, 29), a relatively hydrophobic sequence that has homology with genes encoding herpes simplex type 1 glycoprotein gH, varicella-zoster virus gpIII, and glycoprotein p86 of human cytomegalovirus (7). Antibodies to each of these proteins neutralize virus in the absence of complement, and antibodies to both herpes simplex virus glycoprotein H and varicella-zoster virus gpIII have been shown to block cell fusion by syncytium-forming virus strains (11, 19). Thus EBV gp85 appears to be one of a family of homologous herpesvirus glycoproteins whose role in infectivity may be that of facilitating membrane fusion.

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