Modification of Epstein-Barr Virus Replication by Tunicamycin

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The effect of tunicamycin, which inhibits N-linked glycosylation, on the replication of Epstein-Barr virus was examined. Tunicamycin markedly reduced the yield of virus from producing cells. At concentrations of 1 to 2 μ g of tunicamycin per ml, there was a buildup of intracellular virus in P3HR1-C113 cells but not in MCV5 cells; at a concentration of 5 μ g of tunicamycin per ml in P3HR1-C113 cells, viral DNA synthesis was inhibited as well. Viral glycoproteins lacking N-linked sugars were apparently inserted into the cell membrane, and the small amount of virus made in the presence of drug was able to bind specifically to its receptor on B cells. However, the ability of the virus to induce immunoglobulin secretion by fresh human lymphocytes was impaired. This implies a role for viral glycoproteins in the penetration as well as the attachment of virus.

Epstein-Barr virus (EBV) is a human lymphotropic herpesvirus that causes infectious mononucleosis and has been associated with human malignancies (7). The virus can be derived in cultures from latently infected lymphoblastoid cell lines of human and marmoset origin. Four major virus-specified proteins with approximate molecular weights of 350,000 to 300,000, 250,000 to 200,000, 140,000, and 90,000 to 85,000 are found in the membranes of virus-producing cells and in the mature virion envelope (35). Three of them, gp350/300, gp250/200, and gp90/85, are glycoproteins. The smallest contains N-linked sugars (32), and the two larger molecules share amino acid sequences (12) and are reported to contain both N- and O-linked sugars, with as much as 50% of the molecular mass of gp350/300 being attributed to carbohydrate residues (8, 22). All the glycoproteins have been implicated as being important in viral infectivity. Antibodies to gp350/300 and gp250/200 can inhibit virus binding to lymphocytes (38), and antibodies to all three can neutralize infectivity (11, 33, 36).

The disruption of normal cellular glycosylation pathways interferes with the production and infectivity of several viruses (18), including those of the herpesvirus family (15, 19, 25, 27, 34). In view of the relatively large carbohydrate content of the EBV membrane proteins and the evident importance of the proteins to successful viral replication, we looked at the effects of the inhibition of glycosylation on the production and functions of EBV. We report here on the effects of tunicamycin, an inhibitor of N-linked glycosylation (21).

MATERIALS AND METHODS

Cells. Lymphoblastoid 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 μ g of streptomycin per ml. They included the EBV-receptor-bearing (EBVR⁺) Raji cell line (28), the Daudi cell line (17), which currently does not express EBVR receptors in our laboratory, the superinducible EBV-producing P3HR1-C113 cell line (10), and the marmoset cell line MCV5, which

produces a transforming strain of EBV (the latter two lines were a gift from George Miller, Yale University, New Haven, Conn.).

Induction and harvesting of virus. Producing cells were washed, adjusted to 2×10^6 /ml in RPMI 1640 containing 10% heat-inactivated fetal calf serum, and induced with 30 ng of 12-*O*-tetradecanoylphorbol-13-acetate (TPA) per ml. Cells to be treated with tunicamycin (Sigma Chemical Co., St. Louis, Mo.) were incubated for 3 h with medium containing 1, 2, or 5 μ g of drug per ml before TPA was added. Extracellular virus (ECV) was harvested from spent culture media 3 days after induction with TPA. Media were centrifuged at $4,000 \times g$ to remove cells, and the virus was pelleted by centrifugation at $20,000 \times g$ for 90 min. Pellets were suspended in 1% the original volume of RPMI 1640, centrifuged at $400 \times g$ to remove any residual cells, and filtered through a 0.45- μ m membrane filter (Millipore Corp., Bedford, Mass.). Intracellular virus (ICV) was harvested by lysing cell pellets with two cycles of freezing-thawing and clarifying the lysates at $400 \times g$ to remove intact nuclei.

Assays of viral antigens and virus binding. Viral antigen expression on the surfaces of TPA-induced cells and binding of EBV to EBVR⁺ and non-EBV-receptor-bearing (EBVR⁻) cells were both measured by indirect immunofluorescence on cells fixed with 0.1% *para*-formaldehyde as previously described (31). The antiviral antibodies used included three monoclonal antibodies made against fully glycosylated molecules: A-2-5, which reacts with gp350/300 and gp250/200; G-3-1, which reacts with gp90/85 (32; both were gifts from Bruce Strnad, BBL Research Center, Baltimore, Md.); D4.16, which reacts with gp350/300 (prepared in our laboratory); one monoclonal antibody, BMA 17, made against the bacterium-produced, nonglycosylated polypeptide chain of gp350/300 (a gift from Gary Pearson, Georgetown University, Washington, D.C.); and a high-titered human antibody, B, from a patient with nasopharyngeal carcinoma. Monoclonal antibodies were visualized with fluorescein-conjugated F(ab')₂ sheep antimouse immunoglobulin (Cappel Laboratories, Cochranville, Pa.), and human antiserum was visualized with fluoresceinated staphylococcal protein A (fluorescein isothiocyanate-protein A; Sigma Chemical Co.). Fluorescence intensity and distribution were assessed visually or by analysis of 30,000 cells in an FACS II apparatus (Becton Dickinson and Co., Rutherford, N.J.).

The binding of ³H-EBV was measured by incubating the

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TABLE 1. Effect of tunicamycin on the expression of viral antigens on the membranes of TPA-induced P3HR1-C113 cells as detected by immunofluorescence

Expt	Tunicamycin (μ g)	Intensity of fluorescence determined with indicated antibody (% fluorescent cells)				
		B	A-2-5	D4.16	G-3-1	BMA 17
1	0	+++ (28)	+++ (21)	+++ (24)	++ (16)	ND ^a
	2	++ (29)	+	++ (6)	++ (1)	ND
	5	+	+	+	-(0) ^b	ND
2	0	+++ (29)	+++ (31)	+++ (22)	+++ (23)	-(0) ^b
	2	++ (30)	+	+	+	-(0) ^b
	5	+	+	+	+	-(0) ^b

^a ND, Not done.^b Fewer than 1 of 1,000 positive.

virus with cells for 60 min at 37°C, washing the cells five times, and comparing the acid-precipitable counts remaining attached to cells with the total acid-precipitable counts originally added. EBVR⁻ control cells were included in all binding assays.

Measurement of viral DNA. EBV virion DNA was measured by dot hybridization (14) with the *Bam*HI W fragment cloned in pBR322 (6), which was labeled with [³²P]dCTP by nick translation (29). Samples to be analyzed were digested for 6 h with proteinase K (2.5 mg/ml) at 56°C. The DNA was denatured by the addition of 1/10 volume 5 N NaOH. After incubation at room temperature for 10 min, the samples were neutralized with an equal volume of 2 M ammonium acetate and applied to a nitrocellulose filter with a Manifold apparatus (Millipore Corp.); the filters were then dried in vacuo at 80°C for 2 h. The filters were preincubated for 6 h at 68°C in a hybridization solution containing 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]), 0.2% Ficoll (molecular weight, 400,000), 0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 100 μ g of poly(A) per ml, 100 μ g of calf thymus DNA per ml, and 0.2% sodium dodecyl sulfate. They were then incubated for 18 h in fresh hybridization solution containing 10⁶ cpm of ³²P-labeled probe per ml. The filters were washed successively at 68°C for 30 min each with 2× SSC, 1× SSC, 0.2× SSC, and 0.1× SSC. The filters were air dried and autoradiographed with X-Omat AR film (Eastman-Kodak Co., Rochester, N.Y.).

Total cellular EBV DNA was measured by cell blot hybridization (5). Briefly, samples of 10⁵ cells in phosphate-buffered saline were filtered onto nitrocellulose. The filters were soaked successively in 0.5 N NaOH (7 min), 0.6 M NaCl-1 M Tris (pH 6.8) (three times, 1 min each), and 1.5 M NaCl-0.5 M Tris (pH 7.4) (5 min), air dried, floated on ethanol, air dried, washed twice in chloroform, air dried, rinsed in 0.3 M NaCl, and baked in vacuo at 80°C for 4 h. The dried filters were hybridized with the same probe and in the same manner as the viral DNA blots.

Relative amounts of EBV DNA were determined by integrating scans of autoradiographs of hybridizations. They are expressed in each experiment as a percentage of the values obtained for samples treated with TPA in the absence of any other drug. No hybridization was observed with control samples of herpes simplex virus (HSV) DNA (KOS strain) or calf thymus DNA.

Induction of immunoglobulin synthesis in B lymphocytes. The ability of MCV5 virus to induce immunoglobulin synthesis was measured as previously described (13). Briefly, leukocytes were obtained from heparinized human peripheral blood by flotation on Ficoll-Hypaque and depleted of T cells by forming rosettes with sheep erythrocytes. A total of 200,000 cells were incubated, with or

without virus, in a total volume of 100 μ l in 96-well, round-bottomed tissue culture plates. After 6 days in culture, 100 μ l of medium was added to each well. On day 12, the immunoglobulin concentrations in the media were measured by a double-sandwich micro-enzyme-linked immunosorbent assay (37) with appropriate concentrations of rabbit antihuman immunoglobulin, peroxidase-conjugated rabbit antihuman immunoglobulin, and the substrate hydrogen peroxide with 5-aminosalicylic acid. The colorimetric change was measured at 492 nm. The ability of a monoclonal antibody to inhibit the induction of immunoglobulin synthesis by the virus was measured by preincubating the virus for 1 h at 37°C with antibody F-2-1, which reacts with gp85 (33) (a gift from Bruce Strnad).

Radiolabeling. Cells and virus were labeled extrinsically with ¹²⁵I (Amersham Corp., Arlington Heights, Ill.) by using iodogen (tetrachlorodiphenylglycouril) (9). ¹²⁵I at 0.5 mCi was used for 10⁷ cells suspended in phosphate-buffered saline or for virus pelleted from spent culture media. Proteins of cells and virus were labeled intrinsically with [³⁵S]methionine (specific activity, 1,380 Ci/mmol; Amersham Corp.) or with [³H]glucosamine (specific activity, 40 Ci/mmol; Amersham Corp.). For labeling with [³H]glucosamine, cells were washed 2 days after induction with TPA and suspended at 10⁷ cells per ml in Hanks balanced salt solution supplemented with all nonessential amino acids but with 10% the normal concentration of glucose, 5% dialyzed heat-inactivated fetal calf serum, and the same concentrations of TPA and tunicamycin as were originally added. One hour later, 50 μ Ci of [³H]glucosamine per ml was added, and the cells were harvested 24 h later. For labeling with [³⁵S]methionine, cells were treated in a similar fashion, except that methionine was omitted from the medium, a normal concentration of glucose was added, and 25 μ Ci of [³⁵S]methionine per ml was substituted for [³H]glucosamine. Viral DNA was labeled intrinsically by adding 1 μ Ci of [³H]thymidine (specific activity, 44 Ci/mmol; Amersham Corp.) per ml simultaneously with TPA.

Immunoprecipitation and electrophoresis. Immunoprecipitation was carried out as previously described (31). Briefly, cells were solubilized with lysing buffer and mixed with antibody and protein A-Sepharose CL-4B beads (Sigma Chemical Co.). The precipitates were washed, dissociated by boiling, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20) in 9% acrylamide cross-linked with 0.28% *N,N'*-diallyltartardiamide. Molecular weight markers (Sigma Chemical Co.) were electrophoresed in parallel channels. Gels were stained, destained, infused with 2,5-diphenyloxazole, dried on filter paper, and placed in contact with Kodak XAR film at -70°C for fluorography (4).

TABLE 2. Effect of tunicamycin on the total acid-precipitable radioactivity (counts per minute of [³H]thymidine) associated with ECV pellets and on the proportion of radioactivity capable of binding specifically to EBVR⁺ cells

Expt	Cell line	cpm at indicated tunicamycin concn (μg)							
		0		1		2		5	
		Total ^a	Bindable ^b	Total	Bindable	Total	Bindable	Total	Bindable
1	P3HR1-CI13	2,356	660 (28)	636 (27)	127 (20)	566 (24)	125 (22)	471 (20)	90 (19)
2	P3HR1-CI13	8,550	599 (7)	857 (10)	80 (7)	1,197 (14)	96 (8)	941 (11)	57 (6)
3	P3HR1-CI13	4,582	825 (18)	504 (11)	81 (16)	642 (14)	109 (17)	573 (8)	80 (14)
4	P3HR1-CI13	8,980	1,977 (22)	897 (10)	170 (19)	719 (8)	115 (16)	449 (5)	81 (18)
5	MCUV5	5,196	577 (11)	1,090 (21)	110 (9)	935 (18)	87 (8)	959 (18)	79 (8)

^a Radioactivity harvested in 100 μl. Numbers in parentheses represent the total radioactivity harvested from tunicamycin-treated cells expressed as percentages of the radioactivity harvested from untreated cells.

^b Radioactivity in 100 μl capable of binding to EBVR⁺ cells; no radioactivity bound to EBVR⁻ cells. Numbers in parentheses represent the radioactivity bound to EBVR⁺ cells expressed as percentages of the total radioactivity added.

RESULTS

Effect of tunicamycin on antigen expression in producing cells. P3HR1-CI13 cells were induced with TPA in the presence or absence of tunicamycin. At 3 days postinduction, the expression of EBV antigens in the membranes of cells was determined by immunofluorescence with a panel of antibodies. Tunicamycin had little or no effect on the viability of cells, as judged by the uptake of trypan blue (data not shown), but both the number of cells expressing antigens and the intensity of fluorescence were changed by its presence (Table 1). Reactivity with the polyclonal high-titered human antiserum was least affected, with a small reduction in both the number of cells expressing antigen and the intensity of fluorescence staining. Reactivity with monoclonal antibodies raised against normal or glycosylated molecules was more significantly reduced. Reactivity was never seen with monoclonal antibody BMA 17, which was raised against the totally unglycosylated peptide backbone of gp350/300.

Effect of tunicamycin on the yield of virus from producing cells. Tunicamycin markedly reduced the acid-precipitable tritium counts associated with ECV harvested from both P3HR1-CI13 and MCVU5 cells (Table 2). This apparent reduction in ECV was corroborated by a specific measurement of the relative amount of EBV DNA in similar preparations by blot hybridization (Table 3). Concentrations of 1, 2, and 5 μg of tunicamycin reduced the virus yield from both P3HR1-CI13 and MCVU5 cells and, in MCVU5 cells, also reduced the amount of virus associated with cell lysates from which nuclei had been removed by centrifugation (so-called ICV). At concentrations of 1 and 2 μg of tunicamycin per ml, the reduction in EBV DNA in ECV from P3HR1-CI13 cells

was accompanied by a slight increase in EBV DNA in ICV from P3HR1-CI13 cells. Intracellular EBV DNA in P3HR1-CI13 cells measured by cell blot hybridization mirrored this increase. However, the total amount of extracellular EBV DNA and intracellular EBV DNA made in the presence of tunicamycin was slightly reduced at 1 and 2 μg/ml, and although there was still more intracellular than extracellular EBV DNA at 5 μg/ml, the amount of intracellular DNA was less than that in untreated controls.

Effect of tunicamycin on the ability of virus to bind specifically to receptor-positive cells. Although the yield of virus from TPA-induced cells was considerably reduced by tunicamycin treatment, the drug apparently had little effect on the ability of virus that was made to bind to cell receptors. The proportion of radioactivity associated with ECV from either MCVU5 or P3HR1-CI13 cells that could specifically bind to EBVR⁺ cells was similar in the presence and absence of tunicamycin (Table 2). Specific binding of ECV from P3HR1-CI13 cells treated with 5 μg of tunicamycin per ml was confirmed by immunofluorescence with human antiserum B, the only antibody which reacted significantly with tunicamycin-treated cells, and the relative amounts of virus binding to Raji cells were quantitated by FACS II analysis (Fig. 1). As expected, the yield of bindable ECV was greatest from untreated cells (Fig. 1A), from which more bindable ECV than ICV was harvested. However, despite the reduced yield of virus and the probable reduction in its reactivity with antibody, measurable amounts of bindable virus were clearly harvested from tunicamycin-treated cells (Fig. 1B). The relative amounts of bindable virus measured by this technique were similar to those determined by acid-precipitable counts. More bindable ICV than ECV was harvested from tunicamycin-treated cells, but the relative amounts of ICV in untreated and treated cells were similar (Fig. 1C), indicating that a smaller total amount of virus was made in the presence of tunicamycin.

Effect of tunicamycin on the ability of virus to induce immunoglobulin synthesis by fresh human lymphocytes. Virus derived from P3HR1-CI13 cells lacks DNA sequences necessary for the induction of EBV early antigens in superinfected Raji cells (10). However, live virus from MCVU5 cells is capable of inducing immunoglobulin synthesis by human B lymphocytes. To examine whether virus grown in the presence of tunicamycin retained this capacity, virus from untreated and treated cells was adjusted to contain the same amount of EBV DNA and used to induce immunoglobulin synthesis in fresh human mononuclear cells depleted of T lymphocytes (Table 4). Although similar amounts of bindable virus were added, the ability of the

TABLE 3. Effect of tunicamycin on the relative amount of EBV DNA associated with P3HR1-CI13 cells or with ECV and ICV harvested from MCVU5 and P3HR1-CI13 cells

Tunicamycin (μg)	Amt of EBV DNA in:				
	P3HR1-CI13			MCUV5 ^a	
	ECV ^a	ICV ^a	Cells ^b	ECV	ICV
0	100	65	100	100	56
1	58	91	109	78	49
2	30	79	127	73	21
5	27	47	76	49	8

^a EBV DNA in ECV and ICV is expressed as a percentage of EBV DNA in ECV harvested from untreated cells.

^b EBV DNA in P3HR1-CI13 cells is expressed as a percentage of EBV DNA in untreated cells.

FIG. 1. FACS II fluorescence intensity profile of binding of ECV and ICV from P3HR1-Cl13 cells to Raji cells visualized with B antiserum and fluorescein isothiocyanate-protein A. (A) ECV (—) and ICV (···) from untreated cells. (B) ECV (—) and ICV (···) from cells treated with 5 µg of tunicamycin per ml. (C) ICV from untreated cells (—) and from tunicamycin-treated cells (···). ---, Control profile (no virus).

virus from tunicamycin-treated cells to induce immunoglobulin secretion was significantly reduced. The maximum possible amount of tunicamycin that could have been carried over from treated cells in the most concentrated virus preparations was calculated to be 2% of the original, or 0.025 µg/ml. This concentration did not inhibit immunoglobulin secretion by human lymphocytes in response to pokeweed mitogen (data not shown).

TABLE 4. Ability of virus made in the presence of tunicamycin to induce immunoglobulin synthesis in fresh T-depleted human leukocytes

Virus dilution	Immunoglobulin (ng/ml) induced by virus grown at indicated tunicamycin concn ^a	
	0 µg	5 µg
Stock ^b	12,757	6,329
1/2	13,226	6,087
1/4	9,260	3,074
1/8	6,822	3,570
1/16	4,213	1374

^a The immunoglobulin concentration in the absence of virus was 1,034 ng/ml.

^b Stock virus harvested from treated and untreated cells adjusted to contain equal amounts of EBV DNA.

The binding of EBV to its receptor on B cells is thought to be a function of gp350/300 and gp250/200 (37). These molecules are reported to contain both N- and O-linked sugars. The inhibition of N-linked glycosylation by tunicamycin, which fails to affect virus binding significantly, presumably does not affect any conformation of these two proteins which is necessary for adsorption to occur. Since incomplete glycosylation does, however, block the infectious cycle after adsorption, it implies that EBV glycoproteins play some role in the infection of B lymphocytes beyond that of attachment to the receptor. This supposition receives support from our observations with monoclonal antibody F-2-1, which reacts with gp85 (33). This antibody inhibited the ability of MCV5 virus to induce immunoglobulin synthesis in the absence of complement (Table 5) but had no effect on the ability of virus to bind to B cells, as judged by immunofluorescence or by binding of [³H]thymidine-labeled virus. The specific counts bound to cells after preincubation of virus with antibody F-2-1, with a monoclonal antibody to glycoprotein gB of herpes simplex virus type 2, or with RPMI 1640 were, respectively, 1,975, 1,672, and 1,503.

Effect of tunicamycin on protein and glycoprotein synthesis. P3HR1-Cl13 cells which had been induced in the presence or absence of 5 µg of tunicamycin per ml and labeled with [³H]methionine were lysed and immunoprecipitated with the high-titered human antibody B. The immunoprecipitates were analyzed by SDS-PAGE and autoradiography (Fig. 2). Tunicamycin apparently had little effect on total protein synthesis, although with a long radiographic exposure of the gel to visualize the higher-molecular-weight glycoproteins, a protein in the expected region of gp250/200 was seen early in

TABLE 5. Effect of antibody F-2-1 on the ability of MCV5 virus to induce immunoglobulin synthesis in fresh T-depleted human leukocytes

Expt	Antibody (concn)	Immunoglobulin concn (ng/ml) with:			
		Antibody alone	Virus alone	Antibody + virus	% Inhibition
1	F-2-1 (1/100) ^a	381	1,261	621	51
2	F-2-1 (1/100)	255	3,661	540	85
3	F-2-1 (1/100)	2,110	10,910	4,303	61
4	F-2-1 (1/100)	327	3,790	457	88
1	20×D4 ^b (1/100)	226	1,261	1,465	0
3	20×D4 (1/100)	2,220	10,910	10,155	7

^a This concentration of antibody F-2-1 failed to inhibit the binding of [³H]EBV to EBVR⁺ cells.

^b Hybridoma antibody to HSV gB (2).

untreated cells. Immunoprecipitates from cells labeled with [^3H]glucosamine were also analyzed by SDS-PAGE (Fig. 3). In the absence of tunicamycin, the most prominent glycoproteins immunoprecipitated had molecular weights of approximately 350,000, 250,000, and 60,000 to 85,000. In its presence, they were replaced by one glycoprotein with a molecular weight of approximately 185,000 and one glycoprotein with a molecular weight of approximately 75,000. The larger of these glycoproteins is in the expected size range for a precursor of gp250/200 which lacks N-linked sugars (32). The smaller is apparently present in both treated and untreated cells and may represent a partially glycosylated precursor of gp90/85 or a breakdown product of the higher-molecular-weight glycoprotein. Neither is immunoprecipitated from nonproducing cells (data not shown) so the proteins are presumably not of host origin.

DISCUSSION

Tunicamycin inhibits the cotranslational addition of N-linked oligosaccharide side chains to proteins in mammalian cells, interfering with the formation of dolichol-bound *N*-acetylglucosamine derivatives (21). The drug has been shown to interfere with the biosynthesis of several enveloped viruses, although the consequences of inhibiting glycoprotein formation are variable. Information on its effects on herpesviruses relates principally to HSV. Slightly reduced numbers of enveloped HSV particles are produced in the presence of tunicamycin (15, 34), and these particles are capable of binding to cells (34). However, tunicamycin is reported to inhibit the expression of some viral glycoproteins in infected cell membranes (25), and the infectivity of HSV made in its presence is markedly impaired (15, 19, 27, 34), possibly because of a failure in penetration (34). Our results

FIG. 2. SDS-PAGE of [^{35}S]methionine-labeled peptides immunoprecipitated by antiserum from P3HR1-C113 cells not treated (lanes 1 and 3) or treated (lanes 2 and 4) with 5 μg of tunicamycin per ml. Lanes 3 and 4 are long-term exposures of the gel.

FIG. 3. SDS-PAGE of [^3H]glucosamine-labeled peptides immunoprecipitated by antiserum from P3HR1-C113 cells not treated (lane 1) or treated (lane 2) with 5 μg of tunicamycin per ml.

indicate that tunicamycin has somewhat similar effects on EBV.

The most significant effect of tunicamycin on the biosynthesis of EBV was on the yield of virus from treated lymphocytes. At a tunicamycin concentration of 5 $\mu\text{g}/\text{ml}$, dot blot hybridization showed that approximately 70% less ECV was harvested from P3HR1-C113 cells and 50% less ECV was harvested from MCV5 cells than from controls. The effects of lower concentrations of tunicamycin, which also significantly reduced virus yields, might be accounted for, at least in part in P3HR1-C113 cells, by a failure in the transport of enveloped virus, since the reduction in ECV was accompanied by an approximately equal increase in ICV. However, in MCV5 cells and with a 5- $\mu\text{g}/\text{ml}$ tunicamycin concentration in P3HR1-C113 cells, there was a drop in total intracellular EBV DNA, suggesting that, as noted with HSV (15), the drug has some effect on EBV DNA synthesis.

As expected from previous studies (32), tunicamycin also markedly affected the antigenicity of EBV glycoproteins. The reactivity of producing-cell membranes was lost to two monoclonal antibodies. One of these (G-3-1) has been reported to immunoprecipitate only fully glycosylated proteins from producing cells (32). The other, D4.16, prepared in our laboratory, has similar characteristics. Immunofluorescence with a high-titered human antiserum was, however, still evident at concentrations of tunicamycin that markedly reduced reaction with the monoclonal antibodies. It is possible that the human antiserum contained antibodies to the fourth, unglycosylated EBV membrane protein, p140, and that this accounted for the fluorescence staining. However, p140 has been reported to be expressed only in trace amounts in cells producing EBV (23, 35). Also, the human antiserum could immunoprecipitate glycoproteins from tunicamycin-treated cells, indicating that it is capable of reacting with glycoproteins that lack N-linked sugars. It therefore seems reasonable to conclude that at least the

higher-molecular-weight glycoproteins of EBV can be inserted into the cell membrane in the presence of tunicamycin. At a tunicamycin concentration of 5 µg/ml, there was a significant reduction in the number of cells reacting with the human antiserum as well as with the monoclonal antibodies. This may mean that tunicamycin interferes with the induction of cells into the productive cycle. The reduction in the number of reactive cells was not, however, sufficient to account entirely for the reduction in intracellular and extracellular EBV DNA at this concentration of tunicamycin, indicating that there also had to be a reduction in the amount of EBV DNA made in individual cells. Reactivity was not seen with a monoclonal antibody made against the bacterially produced peptide precursor of gp350/300. This presumably means that this antibody either reacts with an epitope not readily exposed on the surfaces of cells or reacts with an epitope exposed only after a more significant change in conformation than is induced by the loss of N-linked sugars. We were unable, with this antibody, to immunoprecipitate from tunicamycin-treated cells a glycoprotein with a molecular weight as high as that of the glycoprotein immunoprecipitated by the human antiserum from tunicamycin-treated cells (data not shown), so the latter explanation would seem to be the more likely.

Tunicamycin had no effect on the ability of EBV made in its presence to bind specifically to the EBV receptor on B cells, suggesting that, as is the case with HSV (34), there is no apparent requirement for N-linked sugars in the adsorption of virus. However, again in parallel with the effects on HSV (34), tunicamycin treatment did affect later viral functions. EBV made in the presence of tunicamycin had a reduced ability to induce immunoglobulin synthesis in fresh B lymphocytes. This is a function which, for two reasons, can be dissociated from attachment. First, if the virus is irradiated, it still binds to B cells and caps its receptor (3; unpublished data) but fails to induce immunoglobulin secretion. Second, monoclonal antibody F-2-1, which reacts with gp85, fails to inhibit virus binding but is nevertheless capable of neutralizing the induction of immunoglobulin secretion. One of the nucleocapsid proteins of EBV is glycosylated (16), and it is possible that the alteration in the structure of this protein may account for some loss of function of virus made in the presence of tunicamycin. However, it is also tempting to speculate that, as has been suggested for HSV (34), there may be some defect in the ability of virus lacking N-linked sugars to fuse with the plasma membrane either at the cell surface or after endocytosis (24). HSV glycoprotein B (gB) has been implicated in virus penetration (30). The predicted sequence of a protein that may be gp85 (1) is in part similar to the sequence of HSV gB (26), and a monoclonal antibody prepared in our laboratory to HSV gB cross-reacts with EBV gp85 (unpublished data). It then seems feasible that gB and gp85 may perform similar functions.

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