American Journal of Pathology, Vol. 139, No. 1, July 1991

Subcellular Distribution and Life Cycle of Epstein–Barr Virus in Keratinocytes of Oral Hairy Leukoplakia

Jörg-Peter Rabanus,* Deborah Greenspan,* Vibeke Petersen,* Ulrike Leser,† Hans Wolf,† and John S. Greenspan*

From the Oral AIDS Center, Department of Stomatology, School of Dentistry, University of California, San Francisco, California; and Max von Pettenkofer Institute,† Munich, Federal Republic of Germany*

The authors investigated the life cycle of Epstein–Barr virus (EBV) in keratinocytes of oral hairy leukoplakia by combining immunohistochemistry, DNA* in situ *hybridization, and lectin histochemistry with electron microscopy. Diffuse-staining components of the EBV early antigen complex (EA-D), EBV 150-kd capsid antigen (VCA), EBV membrane antigen (gp350/220), and double-stranded DNA were labeled with monoclonal antibodies. An EBV-DNA probe was used to locate EBV DNA. Wheat-germ agglutinin (WGA) was employed to distinguish Golgi-associated compartments. The authors found EBV proteins and EBV DNA only in keratinocytes with apparent viral assembly.* In situ *hybridization showed EBV DNA in free corelike material and in electron-dense cores of mature nucleocapsids. Monoclonal antibodies to nonspecific double-stranded DNA attached to the same structures and to marginated chromatin. Components of EA-D were dispersed throughout the nuclei but accumulated near condensed chromatin and in 'punched-out' regions of the chromatin. Epstein–Barr virus 150-kd capsid antigen was found only in the nuclei, where it appeared preferentially on mature nucleocapsids. As yet unexplained arrays of intranuclear particles that remained unlabeled with all EBV-specific probes reacted intensely with an antiserum against common papillomavirus antigen. Gp350/220 was detectable in various cellular membrane compartments and was highly concentrated on EBV envelopes in peripheral Golgi-associated secretory vesicles. It was less abundant on the extracellular EBV, indicating that viral membrane antigen partly dissociates from the mature virus. Combined lectin-binding histochemistry and electron microscopy demonstrated for the first time that EBV is processed in the Golgi apparatus, which eventually releases the virus by fusion with the plasma membrane. These results provide insight into the biologic events that occur during complete EBV replication* in vivo. *(Am J Pathol 1991, 139:185–197)

Epstein–Barr virus (EBV) infects more than 90% of the adult population worldwide[1] and remains in a fine balance with the immune system for the lifetime of the infected host.[2] Because the virus replicates at a low rate in healthy persons, the site of continuous and controlled EBV production has long remained obscure.

Since the discovery of EBV by Epstein and Barr in 1964,[3] assembly of the virus has been observed only in stimulated or superinfected cultures of latently infected B cells. Recent data, however, suggest that epithelial cells may be primary sites of EBV replication.[4–8]

Human immunodeficiency virus (HIV)-induced depression of the immune system can disturb the equilibrium between EBV and its host.[9] This disequilibrium seems to permit the complete replication of EBV in certain oral epithelial sites, which may lead to the development of hairy leukoplakia, a lesion that most commonly appears on the lateral borders of the tongue of HIV-infected individuals[10,11] (Figure 1).

Electron-microscopic investigations on hairy leukoplakia have provided details about ultrastructural patterns associated with EBV production in permissive cells *in vivo*.[11–19] Immunohistochemistry[11,12] and *in situ* hybridization[20–22] have given insight into the distribution of EBV antigens and DNA in hairy leukoplakia. By themselves, however, these methods are not sufficient to show the assembly and intracellular pathway of EBV as well as the composition of most of the ultramorphologic alterations described in EBV-producing cells. In this study, we

Supported by NIH-PO1-DE-07946 and Deutsche Forschungsgemeinschaft.

Accepted for publication March 7, 1991.

Address reprint requests to Jörg-Peter Rabanus, Oral AIDS Center, Department of Stomatology, School of Dentistry, University of California, San Francisco, CA 94143-0512.

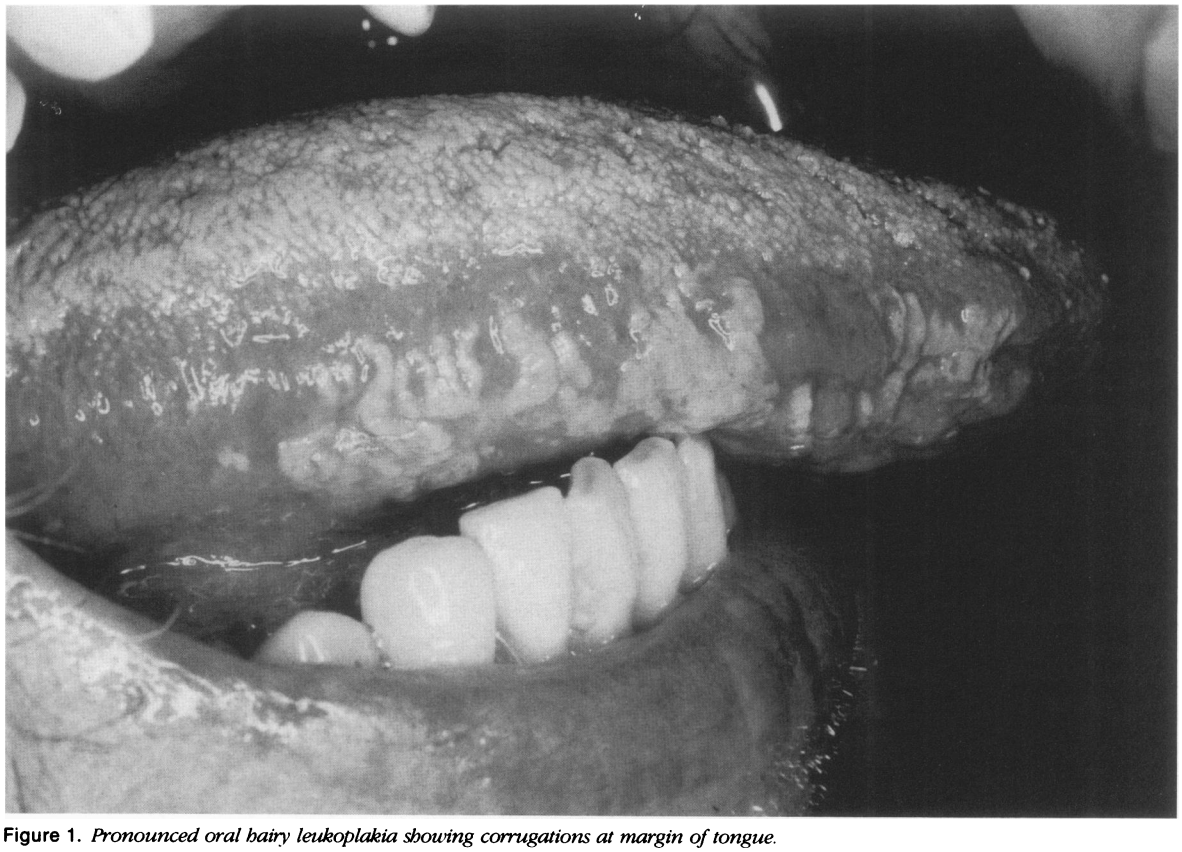

Figure 1. *Pronounced oral hairy leukoplakia showing corrugations at margin of tongue.*

investigated the distribution and the pathway of EBV components by combining electron microscopy with immunohistochemistry, DNA *in situ* hybridization, and lectin histochemistry.

Hairy leukoplakia also exhibits histopathologic features that are similar to those found in human papillomavirus infection and harbors intranuclear particles that resemble papillomaviruses. The positive staining of nuclei in epithelial cells of the upper prickle cell layers of hairy leukoplakia with a rabbit serum against common papillomavirus antigen[10,11,16] and with probes for human papillomavirus DNA[23,24] has been controversial.[12,19,22] We therefore also sought to determine the ultrastructural binding sites for the antiserum against papillomavirus.

Materials and Methods

We obtained punch biopsy specimens of clinically suggested hairy leukoplakia from eight HIV-seropositive men aged 25 to 40 years. As negative controls, we used normal-appearing tissue from the vicinity of the lesions and biopsy specimens of oral gingival warts from two additional HIV-infected patients. All gave informed consent to provide tissue for the study. The biopsy specimens, taken after local infiltration with Xylocaine (Astra, Westboro, MA; 1 ml, epinephrine 1:100,000), were cut into blocks measuring 1 mm × 1 mm × *t* mm (where *t* equals thickness of epithelium) and immediately fixed in 4% paraformaldehyde for 2 hours.

Specimens from each case were prepared by two different procedures. The first procedure involved embedding tissue blocks in methacrylates; several blocks from each case were embedded in Lowicryl K4M (Chemische Werke, Waldkreiburg, FRG) (hydrophilic) and Lowicryl HM20 (hydrophobic). In the second procedure, tissue blocks were infiltrated successively with 10%, 20%, 1.2 mol/l (molar), and 2.3 mol/l sucrose in phosphate-buffered saline (PBS, pH 7.2) and eventually shock-frozen in slush nitrogen (−210°C).

Immunoelectron microscopy and DNA *in situ* hybridization were performed on ultrathin sections of both methacrylate-embedded and shock-frozen specimens from six patients. *In situ* hybridization was also carried out on Epon-embedded (Ladd Research Industries, Burlington, VT) sections from two patients.

Immunoelectron microscopy was performed as described elsewhere.[25] Briefly, nonspecific binding sites were blocked first with 0.1% gelatin and 0.5% bovine serum albumin in PBS, and subsequently with goat nonimmune serum. Next the sections were incubated for 1 hour with affinity-purified monoclonal antibodies (MAb) specific against different epitopes of galactosidase-fused EBV proteins that we produced in *Escherichia coli*. The MAbs were directed against the following antigens: EBV-

membrane antigen gp350/220 of reading frame BLLF1,[26] EBV-capsid antigen p150 of reading frame BcLF1,[27] and EBV-early antigen D p138 of reading frame BALF2.[28] All MAbs were characterized inasmuch as they reacted with cloned recombinant products of the respective proteins.[26–28]

We also applied the following commercially available MAbs against EBV antigens: Anti-EBV VCA IgG1 (MAb 9247/003; DuPont, Billerica, MA) and IgG2a (MAb 9246/003; DuPont), anti-EBV VCA IgG1k (MAb 817, Chemicon, Temecula, CA), anti-EBV EA-D IgG1 (MAb 9240/002; DuPont), and anti-EBV MA IgG1k (MAb 813, Chemicon, El Segundo, CA). In addition, we applied commercially available MAb against double-stranded DNA (IgG2a,k; MAb 030/1613, Chemicon), herpes simplex virus type I IgG1 (MAb 9251/002; DuPont), and cytomegalovirus (CMV) late nuclear protein IgG3 (MAb 9220/4304; DuPont). Finally we concentrated a rabbit antiserum against bovine ('common') papillomavirus antigen (Dako, Santa Barbara, CA) by the factor 10 in Centricon centrifugal microconcentrators (Amicon, Danvers, MA) at 4000 rpm and 4°C in a Sorvall RC2-B centrifuge for 2 hours immediately before the immunoincubation.

The immunoincubated sections were subsequently labeled with affinity-purified goat anti-mouse IgG, goat anti-rabbit IgG, or protein G, conjugated with colloidal gold particles, 1 nm or 5 nm in size (Janssen, Beerse, Belgium). Protein G is a cell wall component of group G streptococci and exhibits IgG-binding properties similar to those of protein A, which is a cell wall component of *S. aureus*.

For indirect lectin-binding histochemistry, we conjugated ovomucoid (Sigma, Type III-O) with 10-nm colloidal gold particles (Janssen, Beerse, Belgium) as described previously.[29,30] We incubated Lowicryl-K4M–embedded sections with a solution of wheat-germ agglutinin (WGA, Sigma) in PBS (100 mg/ml) for 1 hour, and then labeled the lectin-binding sites with the ovomucoid-gold conjugate, applied for 30 minutes.

In situ DNA hybridization was performed with biotiny-

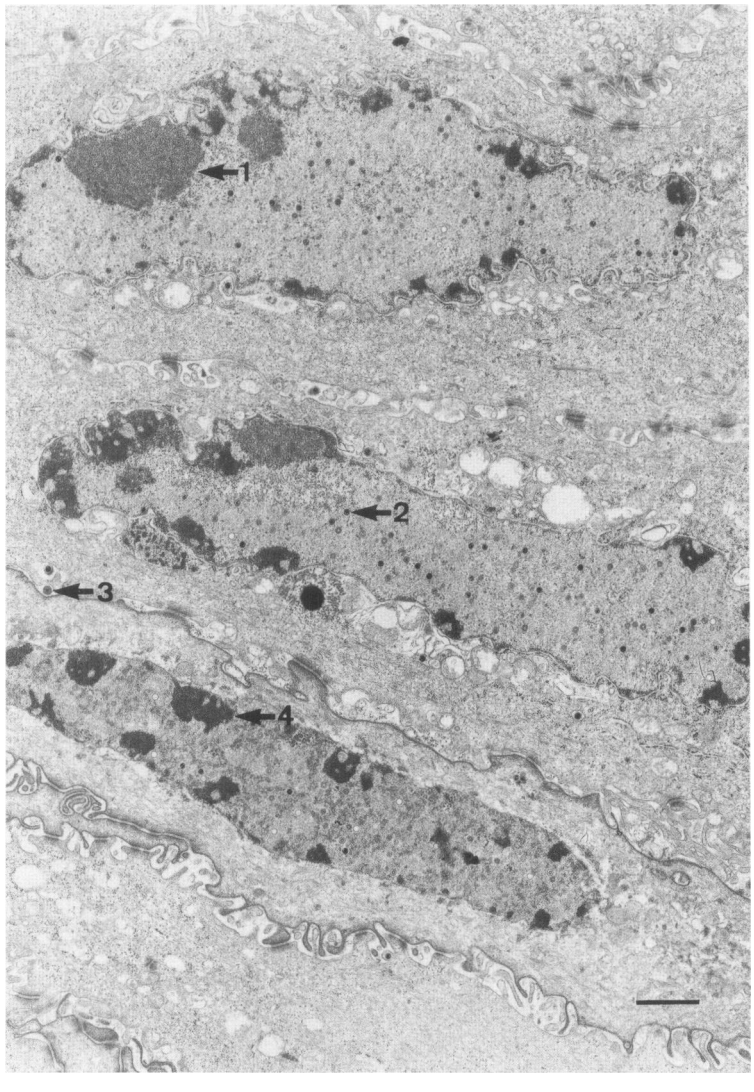

Figure 2. *EBV-producing keratinocytes in upper third of the epithelium of oral hairy leukoplakia. Note the typical ultrastructural appearances:* **(1)** *intranuclear arrays,* **(2)** *nucleocapsids,* **(3)** *extracellular EBV, and* **(4)** *marginated chromatin. Bar represents 1* μ *(×10,000).*

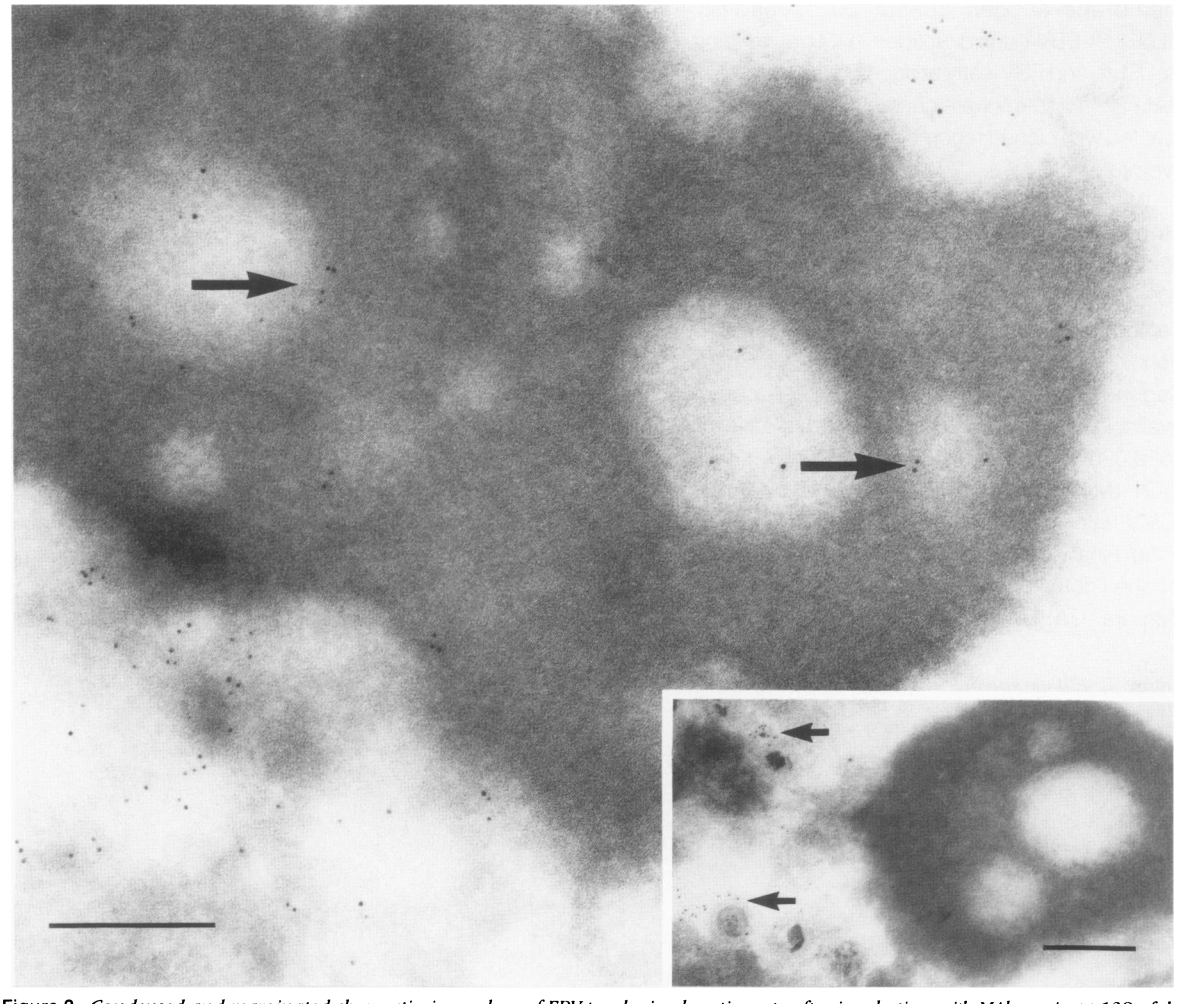

Figure 3. *Condensed and marginated chromatin in nucleus of EBV-producing keratinocyte after incubation with MAb against p138 of the early antigen D complex (EA-D). EA-D has accumulated near marginated chromatin and can be detected in 'punched-out' regions of the chromatin (long arrows).* **Inset:** *Corresponding area after incubation with MAb against virus-capsid antigen (150 kd). The punched-out regions remain without label, whereas mature nucleocapsids are distinctly marked (short arrows). Ultrathin sections of shock-frozen specimens. Bars represent 200 nm (×110,000;* **inset:** *×60,000).*

lated probes on ultrathin sections of shock-frozen, Lowicryl-embedded, and Epon-embedded specimens. The procedure and the biotinylated probes are described elsewhere.[20,21] We used the recombinant plasmid probe pBgl 2-U,[31] which is specific for the large internal repeat sequence of the EBV genome. Tissue-bound probes were labeled with streptavidin conjugated with colloidal gold particles, 5 nm in size (Janssen, Beerse, Belgium).

The sections of shock-frozen specimens were stained with 2% uranyl acetate. The plastic-embedded sections were stained with saturated uranyl acetate and Reynold's lead citrate. All specimens were examined at 80 kV with a Jeol JEM-1200 EX electron microscope.

Results

Of the immunoelectron-microscopic methods applied, the preparation of ultrathin sections of shock-frozen specimens provided the most intense and most specific immunolabeling. Each antigen showed the same distribution in all cases. Epstein–Barr virus proteins and DNA were detected only in keratinocytes with apparent viral assembly in the upper prickle-cell layers of the epithelium (Figure 2); they were not found in the control tissue. Monoclonal antibodies specific to different epitopes of the same protein showed principally the same label pattern, although with varying intensity. The antibodies against herpes simplex virus type I and CMV did not react with either hairy leukoplakia or the control tissue.

The components of the early antigen D complex (EA-D) that were detected with our antibodies were diffusely distributed throughout the nuclei (Figure 3). Early antigen D was found near condensed chromatin and was usually attached to relatively undefined, homogeneous components of various electron densities. Unlike the other antigens examined in this study, EA-D was detected in 'punched-out' regions of condensed and marginated

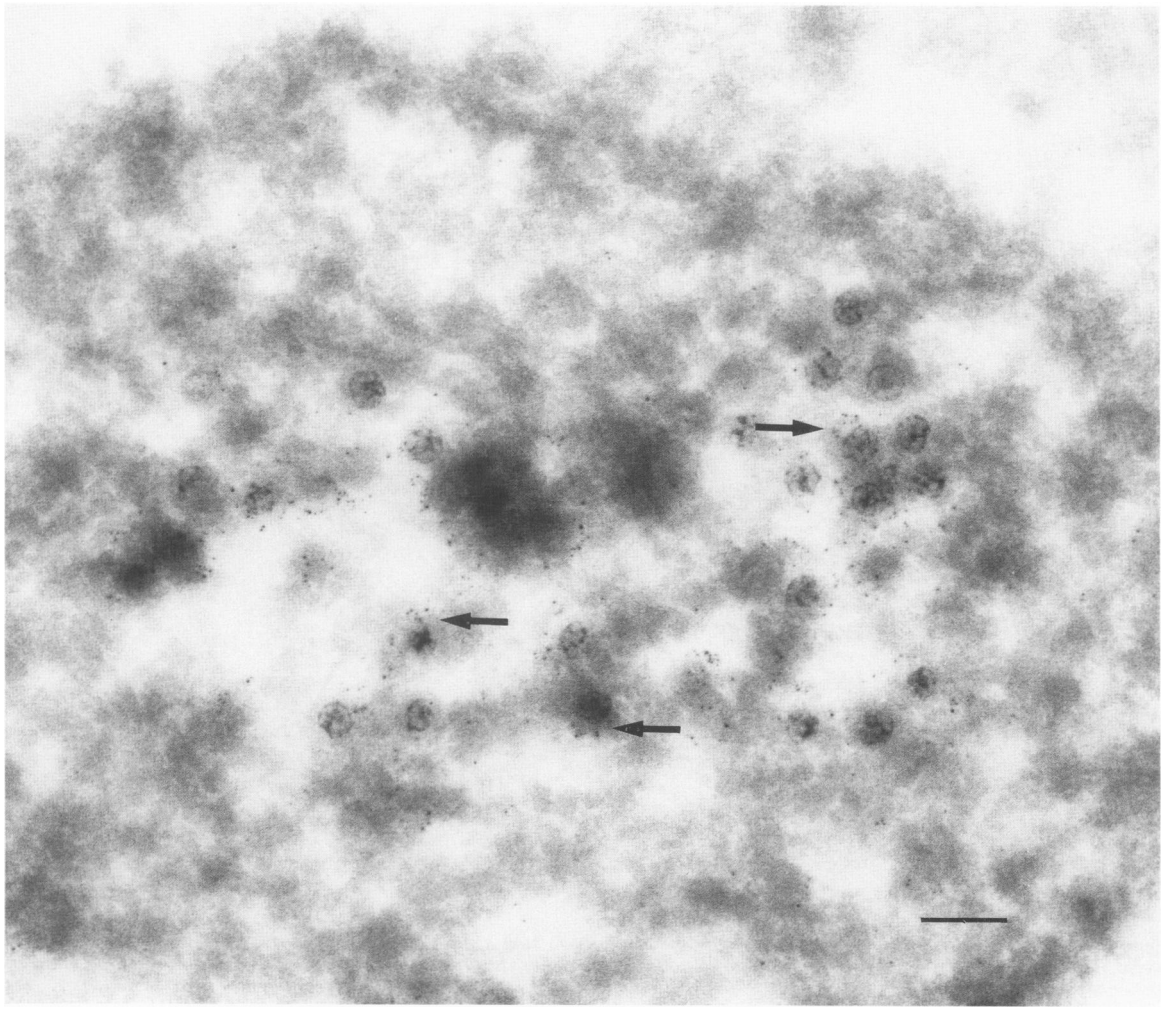

Figure 4. *Portion of nucleus of EBV-producing keratinocyte after incubation with MAb against virus capsid antigen (VCA). Note the association of antigen with mature EBV nucleocapsids (arrows), distinguishable by the higher electron density of their cores. Ultrathin section of shock-frozen specimen. Bar represents 200 nm (×56,000).*

chromatin, common features in EBV-producing keratinocytes of hairy leukoplakia. Early antigen D was not noticed in intranuclear arrays of electron-dense particles, usually about 35 nm in diameter.

Virus capsid antigen (VCA) was found exclusively in the nuclei of EBV-producing keratinocytes. It was mostly associated with morphologically well-defined EBV nucleocapsids at advanced stages of maturation (Figure 4). Once the EBV capsids were enveloped at the nuclear membrane, their VCA was no longer detectable (data not shown). The intranuclear arrays of electron-dense particles were also negative for VCA.

The MAbs against double-stranded DNA reacted with marginated chromatin, cores of viral nucleocapsids, and free, electron-dense intranuclear condensations (Figure 5). Unlike VCA, intraviral DNA was also observed on the shells of less condensed cores of nucleocapsids.

The unembedded and fragile ultrathin sections of shock-frozen specimens lost most of their structure during the procedure of DNA *in situ* hybridization with pBg1 2-U. Therefore we could evaluate only the sections embedded in Lowicryl and Epon. They did not seem to be altered by the hybridization procedure, but showed only weak reactivity with our DNA probes. Epstein–Barr virus DNA was found in the nuclei on condensations resembling free corelike material and occasionally on electron-dense cores of nucleocapsids (Figure 6). Antibody against nonspecific double-stranded DNA also labeled these structures in addition to marginated chromatin (Figure 5).

The antiserum against papillomavirus antigen strongly reacted with the arrays of intranuclear particles (Figure 7B) that remained unlabeled after incubation with the other probes applied in this investigation. The antiserum also reacted with intranuclear structures of keratinocytes in oral warts that we used as control tissue (data not shown).

Membrane antigen (gp350/220) was detected on various membrane compartments of EBV-producing cells. Occasionally we found high amounts of gp350/220 on

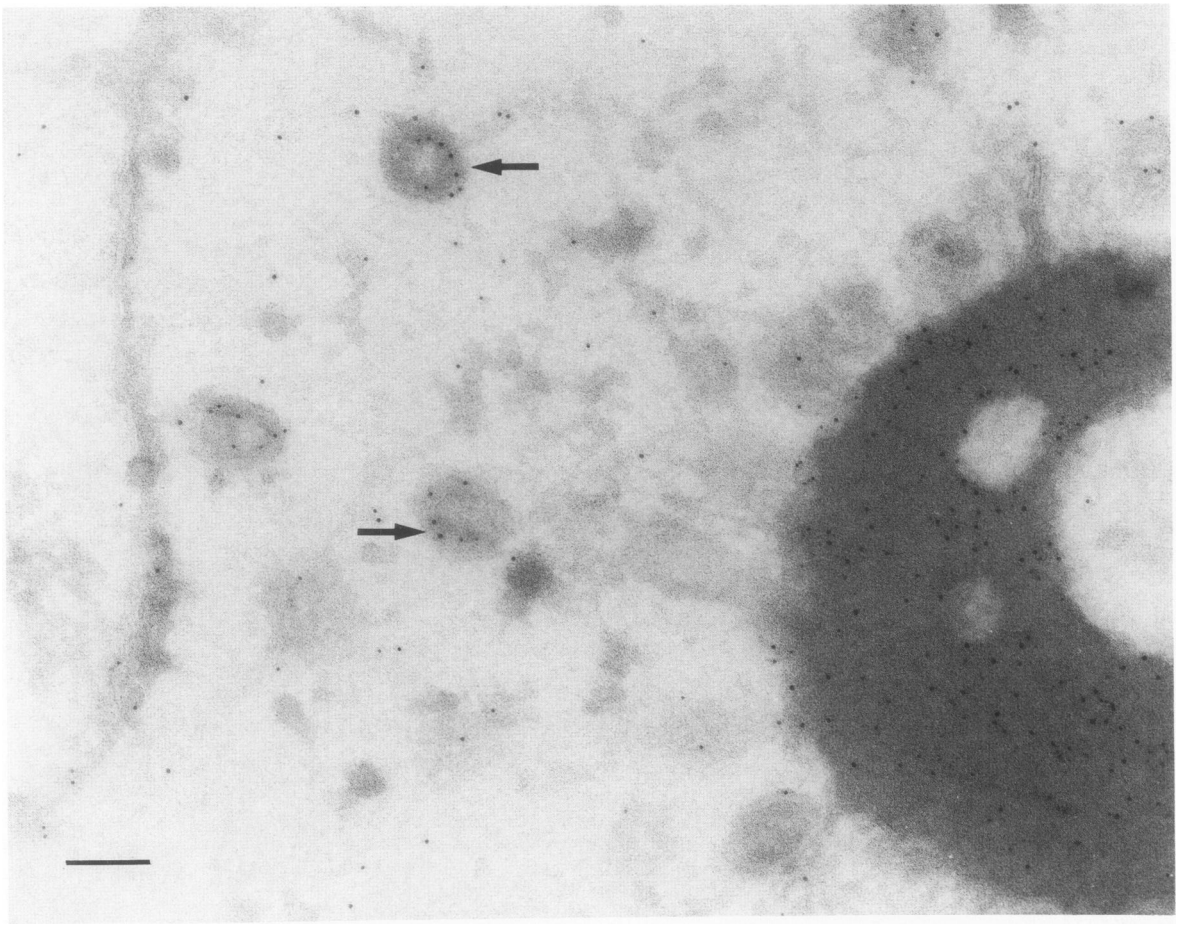

Figure 5. *Nucleus of EBV-producing keratinocyte after incubation with MAb against double-stranded DNA. The chromatin is intensely labeled. Note the shell-like gold label on the core surface of EBV nucleocapsids (arrows). Ultrathin section of shock-frozen specimen. Bar represents 100 nm (×110,000).*

the outside of the nuclear membrane, but it seemed to be more abundant on enveloped EBV capsids that appeared in groups in cytoplasmic vacuoles near the cellular membrane (Figure 8). Most of the extracellular EBV had fewer gp350/220-containing membrane projections on their envelopes than did the cytoplasmic EBV. Gp350/220 could sometimes be detected on the membranes of the vacuoles themselves and on the cell membrane. On rare occasions, small amounts of the antigen were found in the nuclei near the nuclear membrane.

The peripheral cytoplasmic vesicles that contained groups of complete EBV particles were heavily and distinctively labeled with WGA (Figure 9A), which distinguishes them as Golgi-associated secretory vesicles. They released the processed virus by fusing with the plasma membrane (Figure 9B). Envelope and tegument of peripheral intravesicular and extracellular EBV were most intensely labeled with WGA.

Discussion

Oral keratinocytes infected with EBV give rise to the only known lesion in which EBV undergoes complete *in vivo* replication. Because of its clinical appearance, the lesion, which usually appears at the lateral borders of the tongue of severely immunosuppressed persons, has been named oral hairy leukoplakia. Only a few light-microscopic investigations have described the distribution of viral components in hairy leukoplakia.[11,12,20–22] One additional light-microscopic study described the intracellular localization of gp350/220 in the Golgi apparatus.[32] Those data, however, do not provide direct insight into the sequence of viral assembly and the intracellular pathway of EBV components. Several investigators described the ultrastructural alterations specific for EBV-producing keratinocytes in hairy leukoplakia.[11–19] Yet the composition of most ultrastructural features has remained unclear. In this study, combining immunohistochemistry, lectin-binding histochemistry, and DNA *in situ* hybridization with electron microscopy has allowed us to gain direct insight into the molecular composition of some characteristic ultrastructural features of hairy leukoplakia and to correlate them to the distribution and the pathway of EBV constituents.

Viral cores and capsids are assembled in the nuclei of EBV-producing keratinocytes that are found in the upper

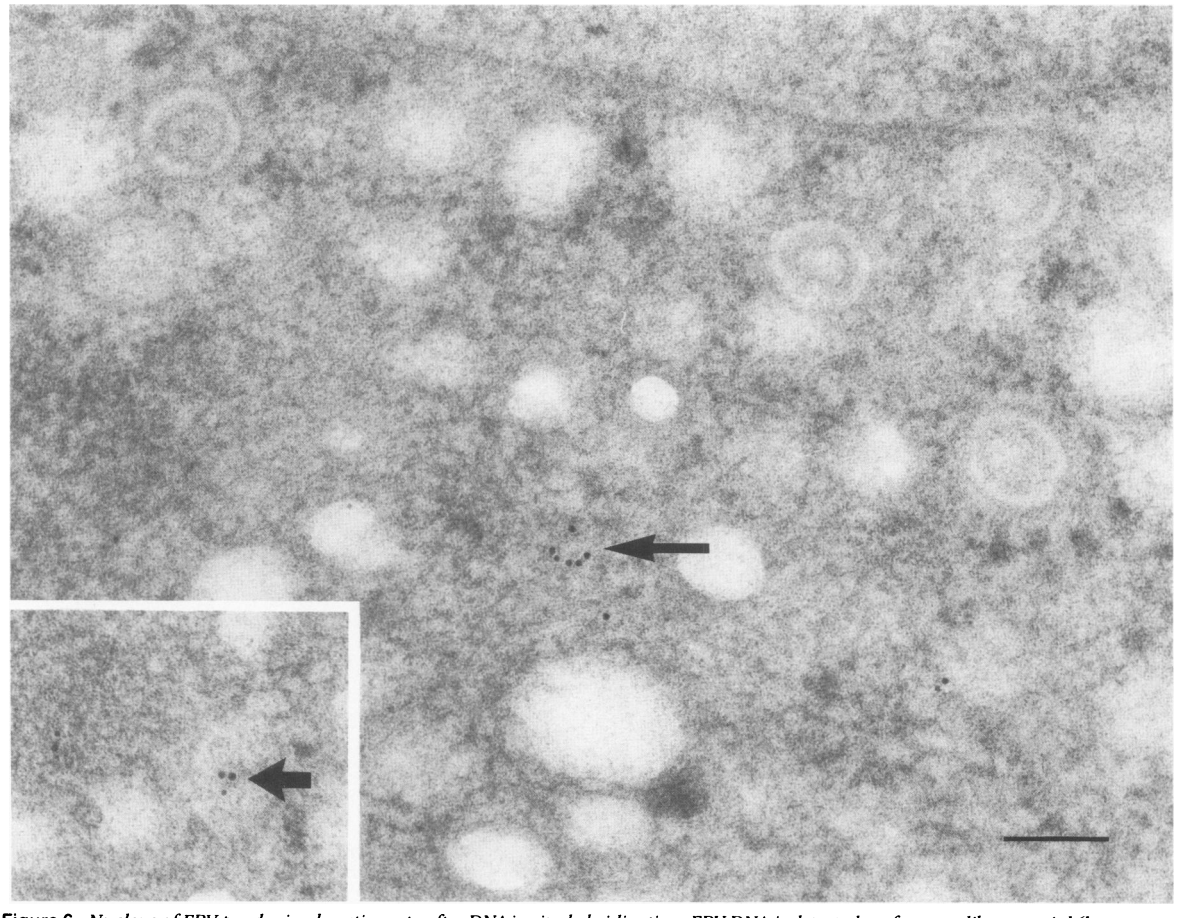

Figure 6. *Nucleus of EBV-producing keratinocyte after DNA* in situ *hybridization. EBV DNA is detected on free corelike material (long arrow) and on electron-dense cores of nucleocapsids (short arrow). Ultrathin sections of Epon-embedded specimens. For both illustrations the bar represents 100 nm (×140,000).*

prickle-cell layers of the epithelium. It might be thought that EBV DNA would be replicated in deeper layers and assembly of the translational products would take place in the upper third of the epithelium. We found three EBV antigens—early antigen, capsid antigen, and membrane antigen—as well as EBV DNA only in EBV-producing cells of the upper third of the epithelium, providing evidence that both replication and assembly are linked to epithelial differentiation.

The plastic-embedded sections prepared for DNA *in situ* hybridization allowed only very weak labeling, as compared with the intensity achieved by light-microscopic techniques.[20–22] The weakness was probably due to the strong cross-linking of already sparse EBV-DNA determinants available in ultrathin sections. The poor labeling obviously does not exclude the occurrence of EBV in lower epithelial layers, although the complete lack of detectable EBV DNA in our system suggests a much lower amount in lower cell layers. This indication of an explosive initiation of a differentiation-linked EBV replication corresponds with previous light-microscopic observations.[20–22] In addition, the labeling was highly specific and appeared mainly on condensations of free core material and electron-dense cores of nucleocapsids.

The MAb against double-stranded DNA applied on ultrathin frozen sections showed the same distribution, in addition to a strong labeling of marginated chromatin. Although labeling of double-stranded DNA would be weak evidence for EBV DNA at the light-microscopic level, the combined observation of the ultrastructurally defined nucleocapsids and double-stranded DNA strongly indicates integrated EBV DNA.

Even though components of the early antigen D complex usually can be found in both the nuclei and the cytoplasm of EBV-producing cells, our MAb reacted only with intranuclear components, as described previously in Raji cells.[33] We found the early antigen p138 rather diffusely distributed throughout the nuclei and, unlike the other viral proteins, accumulated along marginated chromatin and in its punched-out regions. Early antigen induces the replication of the EBV genome and the synthesis of structural viral proteins.[34] Hence the accumulation of p138 at and in cellular chromatin could indicate integrated EBV DNA. Epstein–Barr virus DNA has previously been demonstrated to be integrated into the DNA of Namalwa cells[35] and may be associated with metaphase chromosomes.[36] It has not yet been shown whether p138 binds specifically to EBV sequences, how-

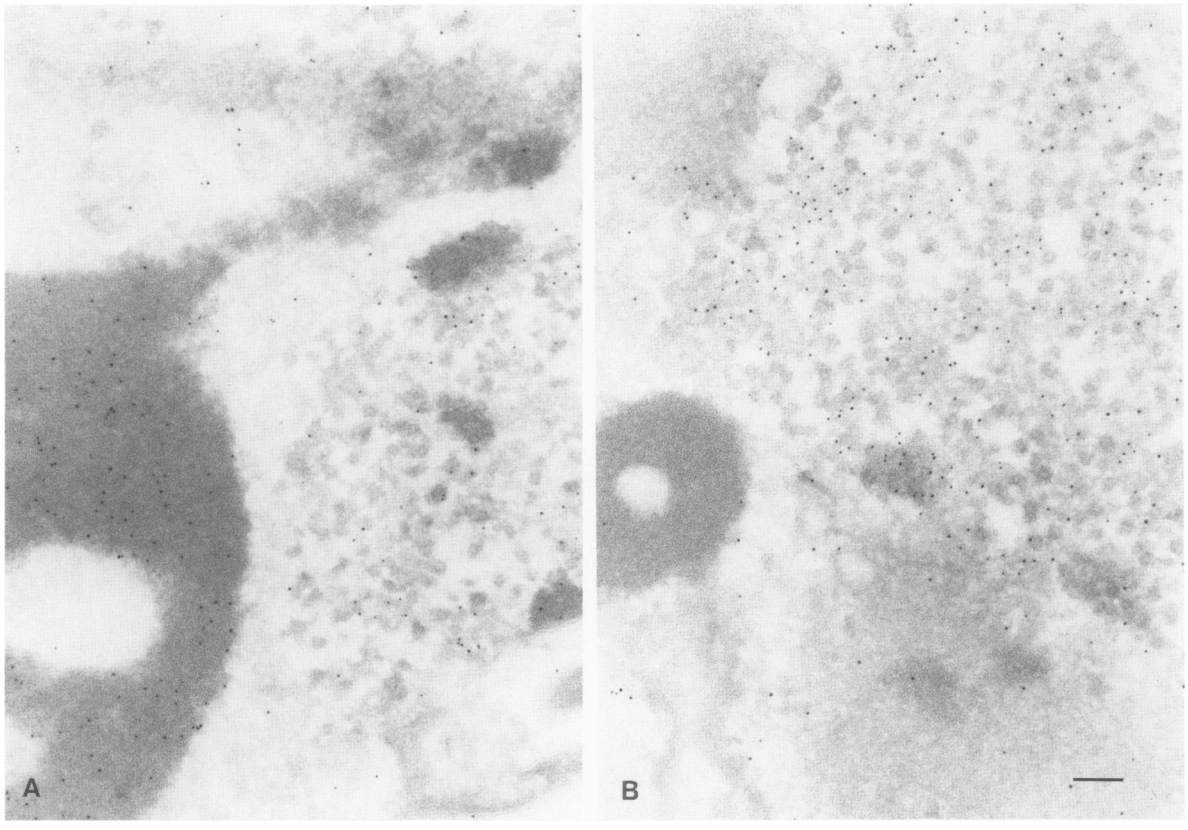

Figure 7. *Nucleus of EBV-producing keratinocyte* (A) *after incubation with MAb against double-stranded DNA and* (B) *after incubation with antiserum against common papillomavirus antigen. Note the specific labeling of the arrayed particles with the antiserum against papillomavirus antigen, whereas the clumped chromatin remains free of label. Ultrathin sections of shock-frozen specimens. For* A *and* B *the bar represents 100 nm (×100,000).*

ever. Hence its peculiar distribution may be attributable to nonspecific binding to components of the host cell chromatin.

Virus capsid antigen was mainly detectable on core-containing nucleocapsids at advanced stages of maturation. Only these mature nucleocapsids are translocated into the cytoplasm by envelopment at the nuclear membrane, as we described recently.[37] Envelopment of herpes simplex virus seems to depend on the completion of DNA packaging and the length of the assembled viral DNA.[38] For EBV, we additionally found here that assembly of the 150-kd capsid antigen seemed to coincide with the final stages of DNA packaging. Hence DNA packaging seems to be crucial for the assembly of VCA, which in turn seems to be important for the envelopment of the completed viral capsids at the nuclear membrane. This notion is supported by the previous observation of empty, thus DNA-free, EBV capsids that accumulated in the nuclei of superficial keratinocytes.[37] Furthermore we found here that enveloped capsids were not labeled with the MAb against VCA, indicating that antigenic sites of the 150-kd protein were shielded by juxtaposing or perhaps even interacting tegument proteins or the envelope.

Unlike VCA and EA-D, membrane antigen (gp350/220) was found mostly on extranuclear enveloped EBV, which frequently collected in groups within cytoplasmic vesicles. We were unable to detect gp350/220 on thickened and reduplicated nuclear membranes, which occur in herpesvirus-producing cells and are thought to harbor virus-specific proteins.[39,40] The fact that the MAb against gp350/220 most strongly labeled EBV envelopes in peripheral cytoplasmic vesicles suggests that the MAb used are directed toward glycosylated epitopes of gp350/220. This is not unlikely, because about 50% of the gp350 mass consists of carbohydrates.[41] It is also consistent with the observations that the glycosylated forms of gp350/220 are stronger immunogens than unglycosylated gp350/220 core proteins,[42] and that our own MAbs failed to react with gp350/220 produced in insect cells (data not shown) that glycosylate proteins differently.

Hitherto it has not been clear how EBV is processed and released by its host cells.[34,39,40] Our data show, unambiguously and for the first time, that EBV follows the same physiologic pathway as cellular proteins during their final processing and egress. In another study, a 165-kd protein was indirectly shown to be processed and glycosylated to gp350/220 in the Golgi apparatus of P3HR-1 cells.[32] It remained unclear, however, whether

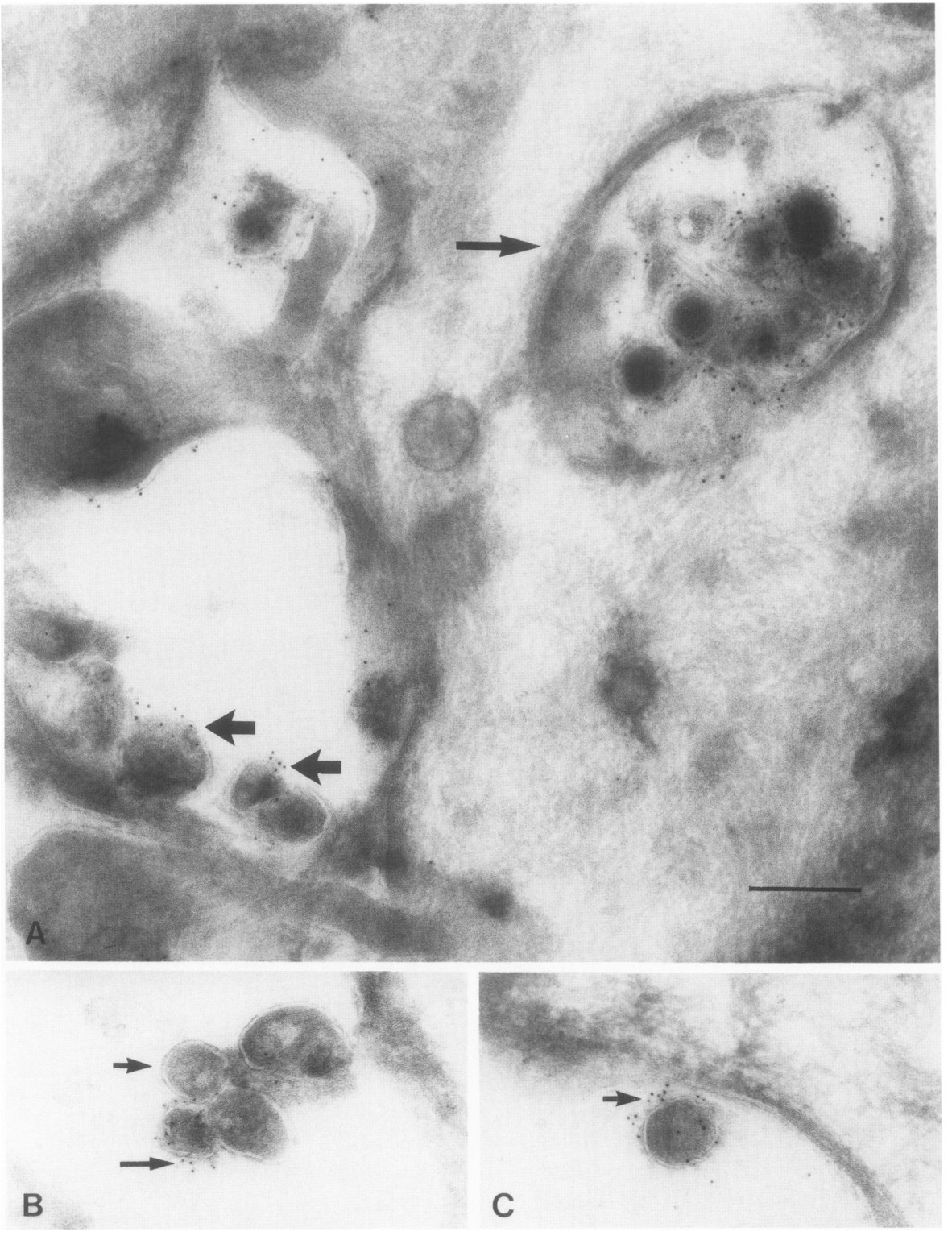

Figure 8. *Periphery of EBV-producing keratinocyte after incubation with MAb against membrane antigen (gp350/220).* A: *Enveloped EBV in the peripheral cytoplasmic vacuole (long arrow) is more intensely labeled than extracellular EBV (short arrows).* B: *Extracellular EBV is labeled exclusively at electron-dense structures projecting out of the viral envelope (long arrow), whereas gp350/220 cannot be detected on EBV without projections (short arrow).* C: *Extracellular EBV seems to be linked to the plasma membrane* via *a gp350/220-positive projection of its envelope (arrow). Ultrathin sections of shock-frozen specimens. For* A, B, *and* C, *the bar represents 200 nm (×90,000).*

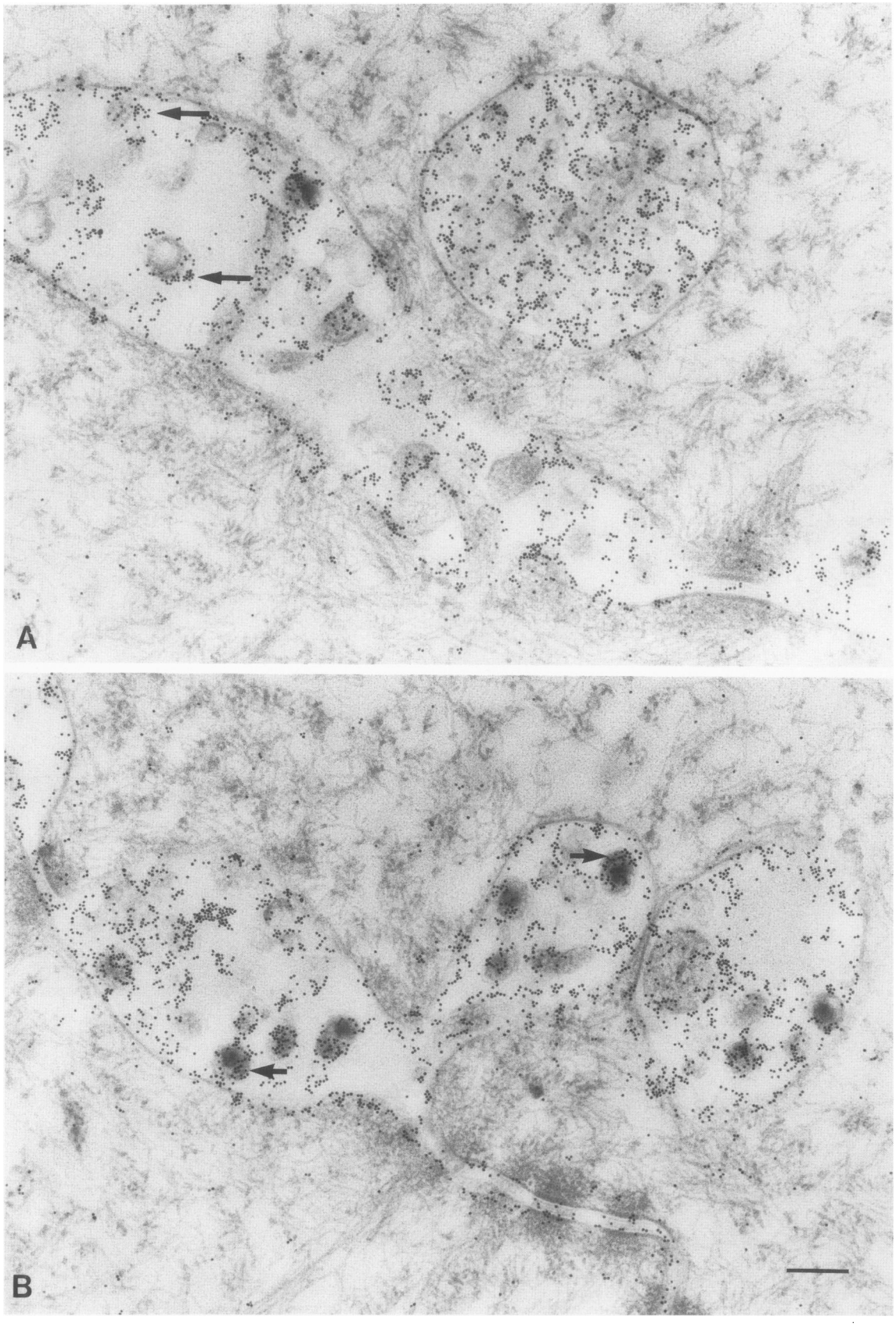

Figure 9. *Periphery of EBV-producing keratinocyte after incubation with wheat-germ agglutinin (WGA) labeled with ovomucoid-conjugated gold (10 nm in diameter). WGA binds to N-acetylglucosamine and stains the Golgi apparatus relatively specifically, but not the endoplasmic reticulum.*[27,36] *EBV appears in groups within peripheral vesicles of the Golgi apparatus* (A), *which eventually open to the cell surface by membrane fusion to release the processed virus* (B). *EBV envelope (long arrows) and tegument (short arrows) are most intensely labeled. Ultrathin sections of Lowicryl-K4M–embedded tissue. For* A *and* B, *the bar represents 100 nm (×53,000).*

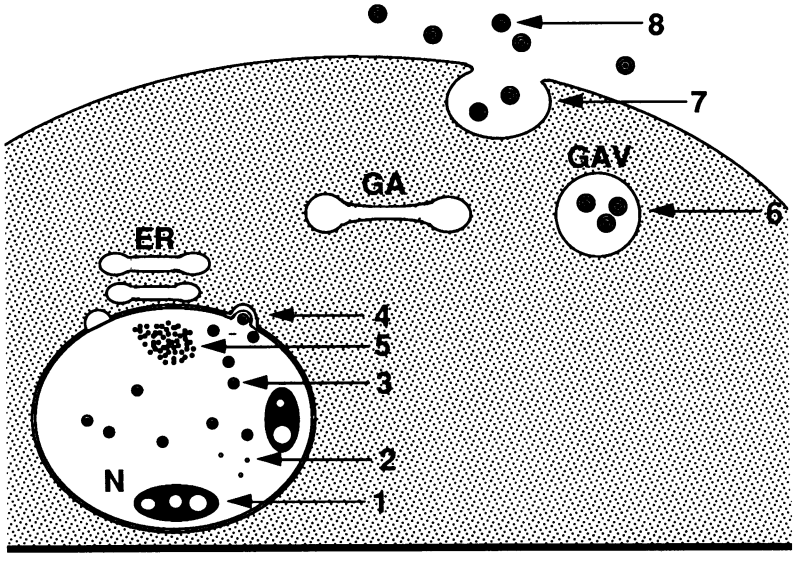

Figure 10. *EBV-producing keratinocyte in oral hairy leukoplakia.* **(1)** *Early antigen was found in punched-out areas of marginated chromatin, which also was intensely labeled for double-stranded DNA.* **(2)** *EBV DNA (large internal repeat) was detected on condensations resembling free core material.* **(3)** *Virus capsid antigen was found on mature nucleocapsids. Their cores were also labeled with MAbs against dsDNA.* **(4)** *After envelopment at the nuclear membrane, virus capsid antigen was obscured by the adjacent membrane.* **(5)** *Intranuclear arrays of electron-dense particles reacted exclusively with an antiserum against papillomavirus antigen.* **(6)** *Enveloped EBV in Golgi-associated vesicles (GAV) reacted with MAb against gp350/220 and wheat-germ agglutinin, which specifically labels the Golgi apparatus and its products by binding to N-acetylglucosamine.* **(7)** *EBV was released by fusion of GAV and the plasma membrane.* **(8)** *WGA stained extracellular EBV most intensely. ER, endoplasmic reticulum; N, nucleus; GA, Golgi apparatus.*

the final processing of gp350/220 precursors took place before or after their integration into the EBV envelopes. In Golgi-associated secretory vesicles of the keratinocytes we examined, gp350/220 was preferentially attached to EBV and only occasionally found on the membranes of the vesicles themselves, whereas virus-free vacuoles did not harbor any detached gp350/220. This indicates that gp350/220 is processed in the Golgi complex of EBV-producing cells after assembly into the viral surface. Our finding that tegument and envelope of EBV particles in peripheral Golgi-associated vesicles were heavily labeled with WGA further supports the idea that EBV gets its final modification in the Golgi apparatus. We also demonstrated that completed EBV is released by fusion of the Golgi-associated secretory vesicles with the plasma membrane. This suggests that egress of EBV depends on physiologic secretory mechanisms of the cell rather than on gp350/220 epitopes that regulate trafficking and release of EBV.[42,43]

Less gp350/220 was detected on extracellular EBV that also lacked surface projections; together these observations indicate that membrane antigen is shed from the virus rather than blocked by diffusing host antibodies. Antibodies directed toward gp350/220 neutralize infectivity of EBV[44] and mediate antibody-dependent cytotoxicity of K cells.[45] Shed viral surface protein might therefore bind and deplete protective antibodies against gp350/220, thus providing a means for EBV to evade the humoral response of the host.

The selective attachment of the MAb to matured viral structures, as demonstrated for VCA and gp350/220, indicates that the viral antigens are continuously processed after their putative production at the ribosomes of the endoplasmic reticulum. Even though these 'mature' EBV proteins are presumably the most immunogenic, some MAb may also be specific to less processed determinants and attach to different ultrastructural features.

Our results provide, for the first time, direct insight into the biology of EBV in its naturally permissive cell, the keratinocyte. As summarized in Figure 10, they disclose parts of the intracellular pathway of EBV, its components, and their relation to typical ultrastructural features of hairy leukoplakia. The composition of some common fine-structural appearances, however, remains unclear. We[10,37] and others[19] previously described arrayed heteromorph and hollow-cored particles in the nuclei of EBV-producing keratinocytes. Similar structures have also been described in cells infected with other herpesviruses[39,46–49] and have been interpreted as partly aggregated capsids.[39] The evidence that these structures are related to herpesviruses is circumstantial, however, and relies heavily on the observation that structures morphologically indistinguishable from small ringlike components have been observed in immature nucleocapsids of herpes simplex virus-1,[50] equine herpesvirus-1,[51] Marek's disease virus, and herpes virus of turkeys.[52] We previously described tubular structures in hairy leukoplakia that were associated with arrays of partially hollow-cored particles.[37] Their proximity and compatible caliber suggest that these tubules may consist of linear aggregations of structural components of the granules. Intranuclear tubular structures also were found in association with intranuclear arrays of other herpesvirus-infected cells[48,49,53,54] and were considered to be a feature of herpesviruses with oncogenic potential,[54] which is consistent with the well-known oncogenicity of EBV. In this study, we observed similar tubular fragments that were interwoven with the papillomavirus-antigen–positive arrays. None of the other probes we used reacted with these ultrastructural features.

The heterogenous probes used in combination with immunoelectron microscopy in this study have given us a better understanding of the biologic events that occur during complete EBV replication in the keratinocyte. Because of the lack of ultrastructural alterations and the low concentration of viral antigens in latently infected cells, immunoelectron-microscopic analysis may not show much beyond that already obtained by light-microscopic investigation. The investigation of the ultrastructural distribution of additional late viral proteins and cellular antigens, however, may further improve our understanding of EBV-cell interactions *in vivo*.

Acknowledgment

The authors thank Evangeline Leash for editing the manuscript and for helpful discussions.

References

1. Wolf H: Virus-assoziierte Krebserkrankungen des Menschen. Mnch Med Wochenschr 1986, 128:633–638
2. Rickinson AB, Yao QY, Wallace LE: The Epstein-Barr virus as a model of virus-host interactions. Br Med Bull 1985, 41:75–79
3. Epstein MA, Barr YM: Cultivation in vitro of human lymphoblasts from Burkitt's malignant lymphoma. Lancet 1964, 1:702–703
4. Young LS, Sixbey JW, Clark D, Rickinson AB: Epstein-Barr virus receptors on human pharyngeal epithelia. Lancet 1986, 1:240–242
5. Shapiro IM, Volsky DJ: Infection of normal human epithelial cells by Epstein-Barr virus. Science 1983, 219:1225–1228
6. Becker J, Leser U, Marschall M, Langford A, Jilg W, Reichart P, Gelderblom H, Wolf H: EB-viral expression depends on the differentiated status of epithelial cells in oral hairy leukoplakia. In Ablashi DV, ed. Epstein-Barr Virus and Human Disease. Clifton, NJ, Humana Press, 1989
7. Wolf H, Haus M, Wilmes E: Persistence of Epstein-Barr virus in the parotid gland. J Virol 1984, 51:795–798
8. Sixbey JW, Lemon SM, Pagano JS: A second site for Epstein-Barr virus shedding: The uterine cervix. Lancet 1986, ii:1122–1124
9. Birx DL, Redfield RR, Tosato G: Defective regulation of EBV infection in patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related disorders. N Engl J Med 1986, 314:874–879
10. Greenspan D, Greenspan JS, Conant M, Petersen V, Silverman S Jr, De Souza Y: Oral "hairy" leucoplakia in male homosexuals: Evidence of association with both papillomavirus and a herpes-group virus. Lancet 1984, 13:831–834
11. Greenspan JS, Greenspan D, Lennette ET, Abrams DI, Conant MA, Petersen V, Freese K: Replication of Epstein-Barr virus within the epithelial cells of oral 'hairy' leukoplakia, an AIDS-associated lesion. N Engl J Med 1985, 313:1564–1571
12. Zhang X, Langford A, Becker J, Rabanus J-P, Pohle HD, Reichart P, Gelderblom H: Ultrastructural and immunohistochemical findings in oral hairy leukoplakia. Virchows Arch [A] 1988, 412:533–542
13. Belton CM, Eversole LR: Oral hairy leukoplakia: Ultrastructural features. J Oral Pathol 1986, 15:493–499
14. Kimmig W, Mensing H, Seyfarth G, Jänner M, Nasemann T: Orale 'hairy' Leukoplakia—Frühsymptom bei HTLV-III/LAV-Infektion. Dtsch Med Wochenschr 1986, 111:1394–1397
15. De Maubeuge J, Ledoux M, Feremans W, Zissis G, Goens J, Andre J, Gourdain JM, Menu R, De Wit S, Cran S, Clumeck N, Achten G: Oral 'hairy' leukoplakia in an African AIDS patient. J Cutan Pathol 1986, 13:235–241
16. Lupton GP, James WD, Redfield RR, Brown C, Rodman OG: Oral hairy leukoplakia. Arch Dermatol 1987, 123:624–628
17. El-Labban N, Rindum J, Nielsen H, Pindborg JJ: Crystalline inclusions in epithelial cells of hairy leukoplakia: A new ultrastructural finding. Scand J Dent Res 1988, 96:353–359
18. Ficarra G, Barone R, Gaglioti D, Milo D, Riccardi R, Romagnoli P, Zorn M: Oral hairy leukoplakia among HIV-positive intravenous drug abusers: A clinicopathologic and ultrastructural study. Oral Surg Oral Med Oral Pathol 1988, 65:421–426
19. Kanas RJ, Abrams AM, Jensen JL, Wuerker RB, Handlers JP: Oral hairy leukoplakia: Ultrastructural observations. Oral Surg Oral Med Oral Pathol 1988, 65:333–338
20. De Souza YG, Greenspan D, Felton JR, Hartzog GA, Hammer M, Greenspan JS: Localization of Epstein-Barr virus DNA in the epithelial cells of oral hairy leukoplakia by in-situ hybridization of tissue sections [Letter]. N Engl J Med 1989, 320:1559–1560
21. De Souza YG, Greenspan D, Felton JR, Hartzog GA, Hammer M, Greenspan JS: Demonstration of Epstein-Barr virus DNA in the epithelial cells of oral hairy leukoplakia [abstr] J Dent Res 1986, 65 (Special Issue):765.
22. Löning T, Heuke P, Reichart P, Becker J: In-situ hybridization to detect Epstein-Barr virus DNA in oral tissues of HIV-infected patients. Virchows Arch [A] 1987, 412:127–133.
23. Eversole LR, Jacobsen P, Stone CE, Freckleton V: Oral condyloma planus (hairy leukoplakia) among homosexual men: A clinicopathological study of thiry-six cases. Oral Surg Oral Med Oral Pathol, 1986, 61:249–255
24. Eversole LR, Stone CE, Beckman AM: Detection of EBV and HPV DNA sequences in oral hairy leukoplakia by in-situ hybridization. J Med Virol 1988, 26:271–277
25. Gelderblom H, Kocks C, L'age-Stehr J, Reupke H: Comparative immunoelectron microscopy with monoclonal antibodies on yellow fever virus-infected cells: Pre-embedding labelling versus immunocryoultramicrotomy. J Virol Methods 1985, 10:225–239
26. Motz M, Deby G, Wolf H: Truncated versions of the two major Epstein-Barr viral glycoproteins (gp250/350) are secreted by recombinant Chinese hamster ovary cells. Gene 1987, 58:149–154

27. Seibl R, Wolf H: Mapping of Epstein-Barr virus proteins on the genome by translation of hybrid selected RNA from induced P3HR1 cells and induced Raji cells. Virology 1985, 141:1–13
28. Motz M, Fan J, Seibl R, Jilg W, Wolf H: Expression of the Epstein-Barr virus 138-kDa early protein in *Escherichia coli* for the use as antigen in diagnostic tests. Gene 1986, 42:303–312
29. Geoghegan WD, Ackerdam GA: Adsorption of horseradish peroxidase, ovomucoid, and anti-immunoglobulin to colloidal gold for the indirect detection of concanavalin A, wheat germ agglutinin and goat anti-immunoglobulin G on cell surfaces at the electron microscopical level: A new method, theory and application. J Histochem Cytochem 1977, 25:1187–1200
30. Virtanen I, Ekblom P, Laurila P: Subcellular compartmentalization of saccharide moieties in cultured normal and malignant cells. J Cell Biol 1980, 85:429–434
31. Desranges C, Bornkamm GW, Zeng Y: Detection of Epstein-Barr viral DNA internal repeats in the nasopharyngeal mucosa of Chinese with IgA/EBV-specific antibodies. Int J Cancer 1982, 29:87–91
32. Bertoni G, Nguyen QV, Humphreys RE, Sairenji T: Intracellular synthesis of Epstein-Barr virus membrane antigen gp350/220. Intervirology 1989, 30:61–73
33. Marschall M: Charakterisierung und Kartierung von Epstein-Barr-Virus Proteinen mit Hilfe von Leserahmen-spezifischen Antiseren. Thesis. Munich, Ludwig-Maximilians-Universität München, 1987.
34. Miller G. Epstein-Barr virus. *In* Flelds BN, ed. Virology. New York, Raven Press, 1985, pp 563–589
35. Matsuo T, Heller M, Petti L, O'Shiro E, Kieff E: Persistence of the entire Epstein-Barr virus genome integrated into human lymphocyte DNA. Science 1984, 226:1322–1325.
36. Harris A, Young B, Griffin B: Random association of Epstein-Barr virus genomes with host cell metaphase chromosomes in Burkitt's lymphoma-derived cell lines. J Virol 1985, 56:328–332
37. Greenspan JS, Rabanus JP, Petersen V, Greenspan D: Fine structure of EBV-infected keratinocytes in oral hairy leukoplakia. J Oral Pathol Med 1989, 18:565–572
38. Vlazny DA, Kwong A, Frenkel N: Site-specific cleavage/packaging of herpes simplex virus DNA and the selective maturation of nucleocapsids containing full-length DNA. Proc Natl Acad Sci USA 1982, 79:1423–1427
39. Roizman B, Furlong D: The replication of herpesviruses. *In* Fraenkel-Conrat H, Wagner RR, eds. Comprehensive Virology 3. Reproduction. New York, Plenum Press, 1974, p 346
40. Dargan DJ: The structure and assembly of herpesviruses. *In* Harris RH, Horne RW, eds. Electron Microscopy of Proteins, Vol. 5, Viral Structure. London, Academic Press, 1986, p 359
41. Morgan AJ, Smith AR, Barker RN, Epstein MA: A structural investigation of the Epstein-Barr (EB) virus membrane antigen glycoprotein, gp350. J Gen Virol 1984, 65:397–404
42. Whang Y, Silberklang M, Morgan A, Munshi S, Lenny AB, Ellis RW, Kieff E: Expression of Epstein-Barr virus gp350/220 gene in rodent and primate cells. J Virol 1987, 61:1796–1807
43. Sairenji T, Bertoni G, Medveczky MM, Medveczky PG, Nguyen QV, Humphreys RE: Inhibition of Epstein-Barr virus (EBV) release from P3HR-1 and B95-8 cell lines by monoclonal antibodies to EBV membrane antigen gp350/220. J Virol 1988, 62:2614–2621
44. Thorley-Lawson DA, Geilinger K: Monoclonal antibodies against the major glycoprotein (gp350/220) of Epstein-Barr virus neutralize infectivity. Proc Natl Acad Sci USA 1980, 77:5307–5311
45. Pearson GR, Orr TW: Antibody-dependent lymphocyte cytotoxicity against cells expressing Epstein-Barr virus antigens. J Natl Cancer Inst 1976, 56:485–488
46. Schaffer PA, Brunschwig JP, McCombs RM, Benyesh-Melnick M: Electron microscopic studies of temperature-sensitive mutants of herpes simplex virus type 1. Virology 1974, 62:444–457
47. Cabral GA, Schaffer PA: Electron microscope studies of temperature-sensitive mutants of herpes simplex virus type 2. J Virol 1976, 48:727–737
48. Atkinson MA, Barr S, Timbury MC: The fine structure of cells infected with temperature-sensitive mutants of herpes simplex virus type 2. J Gen Virol 1978, 40:103–119
49. Stackpole CW: Herpes-type virus of the frog renal adenocarcinoma: I. Virus development in tumor transplants maintained at low temperature. J Virol 1969, 4:75–93
50. Nii S: Electron microscopic observations on FL cells infected with herpes simplex virus 1. Viral forms. Biken J 1971, 14:177–190
51. Perdue ML, Cohen JC, Randall CC, O'Callaghan DJ: Biochemical studies of the maturation of Herpesvirus nucleocapsids species. Virology 1976, 74:194–208
52. Nazerian K, Lee LF, Witter RL, Burmester BR: Ultrastructural studies of a herpes virus of turkeys antigenically related to Marek's disease virus. Virology 1971, 43:442–452
53. Cook MK, Sears JF: Preparation of infectious cell-free herpes-type virus associated with Marek's disease. J Virol 1970, 5:258–261
54. Morgan DG, Achong BG, Epstein MA: Unusual intranuclear tubular structures associated with the maturation of Herpesvirus saimiri in monkey kidney cell cultures. Br J Cancer 1973, 27:434–440