Differentiation-Associated Expression of the Epstein-Barr Virus BZLF1 Transactivator Protein in Oral Hairy Leukoplakia

L. S. YOUNG,^{1*} R. LAU,² M. ROWE,¹ G. NIEDOBITEK,¹ G. PACKHAM,² F. SHANAHAN,² D. T. ROWE,²
D. GREENSPAN,³ J. S. GREENSPAN,³ A. B. RICKINSON,¹ AND P. J. FARRELL²

Department of Cancer Studies, Cancer Research Campaign Laboratories, The Medical School, University of Birmingham, P.O. Box 363, Birmingham B15 2TT,¹ and Ludwig Institute for Cancer Research, St. Mary's Hospital Medical School, London W2 1PG,² United Kingdom, and Oral AIDS Center and Department of Stomatology, School of Dentistry, University of California, San Francisco, California 94143-0512³

Received 6 December 1990/Accepted 7 February 1991

The BZLF1 protein of Epstein-Barr virus (EBV) is a key immediate-early protein which has been shown to disrupt virus latency in EBV-infected B cells. We have generated a monoclonal antibody, BZ1, to BZLF1 which reacts in immunohistology, immunoblotting, and immunoprecipitation and which recognizes both the active, dimeric form and the inactive, monomeric form of the protein. Biopsies of oral hairy leukoplakia, an AIDS-associated lesion characterized by high-level EBV replication, were examined by immunohistochemistry using the BZ1 monoclonal antibody. A differentiation-associated pattern of BZLF1 expression was observed, BZ1 reacting with nuclei of the upper spinous layer of the lesion. This finding suggests that the BZLF1 promoter may be regulated by the degree of squamous differentiation. A comparison of *in situ* hybridization to EBV DNA and viral capsid antigen staining with BZ1 reactivity suggested that BZLF1 expression precedes rampant virus replication. The inability to detect EBV in the lower epithelial layers of oral hairy leukoplakia raises questions concerning the nature of EBV latency and persistence in stratified squamous epithelium.

Oral hairy leukoplakia (OHL) is a recently described epithelial lesion occurring in association with AIDS (7). OHL presents as raised white areas usually on the lateral border of the tongue. The histological appearance is characterized by an intracellular edema of spinous cells, alterations of the nuclei such as basophilic nuclear inclusions, and superficial parakeratosis (11). Although this histology resembles that observed in papillomavirus-associated infections, there has been no reliable demonstration of such an infection in OHL. Initial examination of OHL under the electron microscope showed the presence of a herpes-type virus particle which was later confirmed as Epstein-Barr virus (EBV) both by DNA hybridization studies and by immunohistochemical analysis with antibodies directed against EBV antigens of the virus lytic cycle, e.g., viral capsid antigen (VCA) and early antigen (EA-D and EA-R) (8). On treatment with acyclovir, an inhibitor of the herpesvirus DNA polymerase, OHL was found to regress (4), implicating EBV as an etiological agent in the development of this nonmalignant epithelial proliferation.

In situ hybridization to EBV DNA has revealed replicating EBV distributed focally in the more differentiated layers of OHL (2, 11). This is in agreement with the pattern of immunostaining observed by using either polyclonal human sera to late viral antigens or monoclonal antibodies (MAbs) to VCA, early antigens (EA-D and EA-R), and membrane antigen. The association of EBV replication with the terminally differentiating tongue epithelial cells of OHL is consistent with previous studies of epithelial EBV infection (23) and suggests that OHL may represent an exaggerated form of the natural epithelial cell infection with EBV.

In this study we have generated a MAb to the key immediate-early protein BZLF1, which has been shown to disrupt virus latency in B cells (12). This MAb has been fully

characterized and used to analyze the pattern of BZLF1 expression in OHL. We demonstrate, for the first time, the differentiation-associated expression of BZLF1 in a biologically relevant cellular environment, i.e., human epithelial cells.

MATERIALS AND METHODS

Collection of OHL biopsies. The subjects chosen for tongue biopsies were individuals with symptomatic human immunodeficiency virus disease (CDC IV) in whom OHL had persisted for at least 3 months. Subjects were evaluated for the presence of OHL by two physicians before being submitted to tongue biopsy. Under local anesthetic, an elliptical incisional biopsy (2 mm wide by 3 mm long) was taken from the side of the tongue with OHL. The tissue sample was snap-frozen in liquid nitrogen and subsequently stored at -70°C. Four of the biopsies analyzed were from British patients, and two biopsies were from American patients.

Generation of MAbs. A fusion protein containing part of *Staphylococcus aureus* protein A at its N terminus joined to the full-length BZLF1 sequence (pREX-BZLF1) was used for immunization (3). Hybridomas were generated as previously described (19), and culture supernatants were screened for relevant antibodies by using an enzyme-linked immunosorbent assay (ELISA) with a different BZLF1 fusion protein (pEX-BZLF1), containing part of the CII protein of phage λ fused to BZLF1, as the positive target antigen and with phage lambda control protein (NpEX) as an irrelevant target antigen (17). The hybridoma cultures that produced apparently BZLF1 specific antibodies in the ELISAs were subsequently tested for reactivity on virus-productive and nonproductive cell lines in an indirect immunofluorescence test. At this stage, hybridomas of interest were recloned by limiting dilution and characterized further by immunoblotting experiments. In this way an immunoglobulin

* Corresponding author.

lin (Ig) G1 MAb, designated BZ1, specific for BZLF1 was isolated.

Immunofluorescence and immunohistochemical staining of cells and tissues. For immunofluorescence, a three-step double-conjugate indirect method was used. Methanol-fixed cell spreads or cryostat sections were rehydrated with phosphate-buffered saline (PBS) containing 20% normal rabbit serum and 10% EBV-seronegative human serum and incubated with various dilutions of hybridoma culture supernatant for 2 h at 37°C. Bound MAb was detected by incubation with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (Sigma) for 1.5 h at 37°C, washing in PBS, and then incubation for 1 h at 37°C with FITC-conjugated rabbit anti-goat IgG (Sigma). The slides were finally washed in PBS, mounted in DABCO (1,4-diazabicyclo[2,2,2]octane)-based antifading mountant, and visualized by fluorescence microscopy. For double staining, an indirect immunofluorescence technique was used. This procedure relied on the different subclass specificities of the primary MAbs and used a mixture of FITC-conjugated goat anti-mouse IgG1 and rhodamine-conjugated goat anti-mouse IgG2a (Southern Biotechnology Associates) as the second step. Immunohistochemistry was performed by the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique as previously described (1).

In situ hybridization. In situ hybridization was performed as described previously (13), using the ³⁵S-labeled plasmid pBa-W (3.1-kb *Bam*HI-W internal repetitive fragment of EBV strain M-ABA, subcloned in pBR322) kindly provided by G. W. Bornkamm, Munich, Germany (16). In brief, frozen sections were fixed in 4% paraformaldehyde for 20 min at room temperature (RT), incubated in 0.02 N HCl for 10 min at RT, and then subjected to a proteolytic digestion in 0.125 mg of pronase (Boehringer, Mannheim, Germany) per ml for 10 min at RT. After extensive washing in PBS, 25 µl of hybridization mixture containing 1 ng of the labeled probe was applied to a section. Cell and probe DNA were denatured together for 2 min at 95°C, and hybridization was carried out overnight at 37°C. Slides were then washed in 50% formamide–0.1× SSC (SSC is 0.15 M NaCl plus 0.015 sodium citrate)–10 mM dithiothreitol for 4 h, rinsed in 2× SSC, dehydrated through graded ethanols, dipped in Ilford G5 emulsion, exposed at 4°C, developed, and counter-stained.

Double-labeling immunohistochemistry and in situ hybridization. For the simultaneous detection of EBV DNA and the BZLF1 protein in the same tissue section, frozen sections were fixed sequentially in acetone and chloroform for 30 min each at RT. Immunohistochemistry was carried out as described above. After APAAP staining, slides were subjected to in situ hybridization without any additional treatment. After hybridization overnight and washing as described above, slides were air dried briefly, dipped into Ilford G5 emulsion, and exposed for 3 days. The slides were then developed, counterstained with hematoxylin, and embedded with glycerol gelatin (Dakopatts, Glostrup, Denmark).

SDS-PAGE and immunoblotting. Protein extracts of appropriate cell lines were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) on 10% polyacrylamide Laemmli gels, blotted onto nitrocellulose filters, probed with specific antibodies, and detected with ¹²⁵I-protein A, all as described previously (20).

Epitope mapping of the BZLF1-specific MAb. Various deletion and point mutants of BZLF1 were transcribed and translated in vitro. The radioactive protein (labeled with [³⁵S]cysteine) was then immunoprecipitated with the

BZLF1-specific MAb BZ1, and 30 µl of in vitro translation reaction mixture was mixed with 25 µl of BZ1 culture supernatant and 220 µl of ELB (250 mM NaCl, 50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.0], 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM dithiothreitol, 0.1% Nonidet P-40) at 4°C for 30 min. Then 12.5 µl of a 1:1 slurry of protein A-Sepharose beads that had been precoated with a rabbit anti-mouse Ig antibody (Z259; Dakopatts) was added, the solution was mixed for 30 min at 4°C, and the beads were collected by centrifugation and washed three times in ELB. The bound proteins were eluted in SDS-gel sample buffer and analyzed on a 17.5% polyacrylamide gel. The protein A beads were precoated by mixing 200 µl of a 1:1 slurry of beads in ELB with 12 µl Z259. After 30 min at 4°C, the beads were washed three times in ELB.

Supershift experiment to demonstrate that the BZLF1-specific MAb recognizes the active dimer of BZLF1. Fusion proteins of *Sma*I-C BZLF1 cloned at the *Bam*HI-*Eco*RI sites of pGEX and a two-repeat clone of the EBNA-LP (as a control) were induced and purified by chromatography on glutathione agarose. The concentration of each fusion protein was determined by estimating the intensity of the Coomassie-stained gel band on a polyacrylamide gel in comparison with known amounts of bovine serum albumin.

Standard gel retardation reactions were performed as described previously (3). Each reaction mixture contained 6 ng (=0.5 pmol) of ³²P-labeled M 19-mer (double-stranded GAAGCACTGACTCATGAAG), 100 µg of poly(dI-dC) per ml, and, where indicated, 0.1 µg (=1 pmol) of fusion protein, 1 µl of BZ1 ascites fluid, or 1 µl of PE2 (a MAb to EBNA2; 22) ascites fluid. The fusion protein was allowed to bind to the radioactive DNA for 20 min at RT prior to addition of the appropriate antibody and incubation for a further 20 min. Controls were incubated for the same length of time. Complexes were resolved by gel electrophoresis on a 4% polyacrylamide gel in 0.5× Tris-borate-EDTA and were detected by autoradiography.

RESULTS

Characterization of the BZLF1-specific MAb (BZ1). An IgG1 hybridoma (designated BZ1) was isolated which stably produced antibody specifically reactive against productively infected EBV cell lines, as assessed in immunofluorescence staining. This MAb reacted strongly in immunoblotting with the B95.8 and P3HR1 producer cell lines but not with cell lines in which EBV is latent (i.e., X50-7 and Raji) (Fig. 1). Furthermore, no reactivity was observed in EBV-negative cell lines. The major protein recognized by BZ1 in B95.8 migrated with an *M_r* of about 39,000, whereas that observed in the P3HR1 cell line migrated with an *M_r* of approximately 42,000. This result is in agreement with previous studies using either polyclonal human sera or a rabbit monospecific antiserum (17, 18). The specificity of BZ1 was further demonstrated by the reactivity of the MAb with 293 cells transfected with BZLF1 cDNA and not with control transfectants (Fig. 1).

To map the epitope recognized by BZ1, we used a range of BZLF1 deletion mutants (Fig. 2a) cloned into the transcription vector SP64. When linearized and transcribed with SP6 polymerase, RNA was produced which yielded BZLF1 proteins of the expected size when translated in the reticulocyte lysate (Fig. 2b). MAb BZ1 was then used in immunoprecipitations of these proteins in vitro translated in the presence of [³⁵S]cysteine. Whereas the N-Pst1 deletion

FIG. 1. Immunoblot showing the reactivity and specificity of MAb BZ1 with BZLF1 protein in productively infected cell lines (B95.8 and P3HR1) and BZLF1 transfectants (293 + BZ). No reactivity was observed with EBV-positive latently infected EBV-positive lines (X50-7 and Raji), an EBV-negative line (BL40), or control transfectants (293 + pUC).

mutant of BZLF1 failed to be recognized by BZ1, all of the other deletion mutants were readily detected (Fig. 2c and d). This finding suggested that the epitope recognized by BZ1 resided in the dimerization domain of BZLF1. Point muta-

tions within the dimerization region did not affect BZ1 recognition. The M point mutant was immunoprecipitated efficiently in two such experiments (one illustrated Fig. 2c), even though its precipitation seems to be reduced in Fig. 2d.

The KL mutant is unable to dimerize under these conditions (9), so the reactivity of BZ1 with the dimerization domain of BZLF1 and the efficient recognition of these mutants raised the question of whether this MAb would recognize the dimerized, functional form of the protein (10) or might be reacting only with the inactive, monomeric form. To examine this point, gel retardation assays were performed using the AP-1 site upstream of BMLF1 as the target and glutathione S-transferase (GST) fusion proteins of either BZLF1 (GST-SmaI-C) or, as a control, EBNA-LP (GST-SmaI-C) to the AP-1 site, as shown previously (Fig. 3, tracks 7 to 9). Addition of MAb BZ1 to the gel retardation assay shifted the BZLF1-DNA complex (track 8), indicating that the MAb was able to recognize the functional active dimerized form of the BZLF1 protein. No effect was observed with use of a MAb against the EBNA2 protein (track 9), and no shift was seen with the GST-LP fusion protein (tracks 4 to 6).

Reactivity of BZ1 with OHL biopsies. The OHL biopsy material was initially screened for EBV infection by in situ hybridization to viral DNA and by immunostaining for VCA. Six biopsies were selected for subsequent analysis. Serial sections were then analyzed by in situ hybridization and stained with MAb BZ1 and the anti-VCA MAb V3 (Fig. 4). In situ hybridization with an EBV-specific probe resulted in

FIG. 2. Epitope mapping of MAb BZ1. (a) Diagrammatic representation of the various deletion and point mutations used; (b) in vitro translations without immunoprecipitation; (c and d) two experiments with immunoprecipitation.

FIG. 3. Supershift experiment demonstrating that MAb BZ1 recognizes the active dimer of BZLF1. The gel retardation reactions were performed as previously described (2), using the AP-1 site upstream of BMLF1 as the target. Tracks: 1 to 3, reaction mixtures containing no fusion protein; 4 to 6, reaction mixtures containing EBNA-LP (2R) fusion protein; 7 to 9, reaction mixtures containing BZLF1 *Sma*-C fusion protein; 2, 5, and 8, incubated with the MAb BZ1; 3, 6, and 9, incubated with the EBNA2-specific MAb, PE2.

an accumulation of grains over nuclei of the upper layers of the epithelium (Fig. 4a). This signal was usually very intense, leading to saturation of the emulsion even after short exposure times (2 to 3 days). Several clusters of positive cells separated by apparently noninfected cells were identified in each specimen, resulting in a focal distribution of EBV replication. Occasional cells adjacent to foci of viral replication displayed a weaker signal. No signal was observed over the basal cell layers, and no signal was obtained with unrelated probes (not shown).

The reactivity of both MAb BZ1 and V3 was consistent with this pattern of viral replication. Immunohistology with BZ1 MAb produced an intense staining of nuclei in the upper layers of OHL epithelium (Fig. 4b). Interestingly, the stratum corneum demonstrated a markedly reduced level of EBV replication as assessed by all three criteria, i.e., in situ hybridization, BZ1 staining, and V3 staining.

To assess the relative association of BZLF1 and VCA expression, double immunofluorescence staining was performed. In these experiments, colocalization of BZLF1 and VCA proteins was observed in certain cell layers (Fig. 5). However, a population of cells that were BZLF1 positive but VCA negative were also observed. These cells were present in the upper spinous layer and generally appeared as a single cell layer immediately preceding VCA expression. This observation was confirmed in experiments in which sections of OHL were stained with MAb BZ1 and then hybridized for EBV DNA (Fig. 4c and d). This approach permits localization of antigen and viral DNA on a single section. A layer of BZLF1-positive cells displaying a weak EBV-specific signal was observed below cell layers in which the strong signal generated by high levels of virus DNA obscured BZ1 staining. No BZ1 staining was observed lower in the epithelium.

DISCUSSION

We have generated a specific MAb, BZ1, against the BZLF1 protein which reacts in immunohistology, immunoblotting, and immunoprecipitation assays. Apart from the usefulness of BZ1 in the analysis of BZLF1 expression in cells and tissues, the ability of this reagent to recognize the

active, dimeric form of the BZLF1 protein suggests that it will also be important in functional studies.

Epitope mapping experiments localized the region of BZLF1 recognized by BZ1 to the dimerization domain. Supershift assays clearly demonstrated that BZ1 recognized the functional dimerized form of BZLF1. However, point mutations which prevent the homodimerization of BZLF1 did not prevent recognition of the protein by BZ1, suggesting that the MAb reacts with both the active, dimeric form and the inactive, monomeric form of the protein. Thus, BZ1 must recognize either (i) an epitope within the dimerization domain which does not interfere with the functional dimerization of BZLF1 or (ii) an epitope outside of the core dimerization domain. These possibilities are currently being investigated by using other mutants of BZLF1.

The reactivity of BZ1 with nuclei of the upper spinous layer of OHL confirms previous studies demonstrating a differentiation-associated pattern of EBV replication in this lesion (11, 23). The influence of squamous differentiation on BZLF1 expression may be controlled at the BZLF1 promoter level by differentiation-associated regulatory factors. BZLF1 appears to be expressed prior to rampant virus replication, as assessed either by double staining for BZLF1 and VCA or by in situ hybridization and BZLF1 staining. Interestingly, the superficial parakeratotic layer appears to have a markedly reduced level of both BZLF1 expression and viral DNA. This probably results from the reduced availability of viral antigens and DNA in the nuclei of this hyperparakeratotic layer, since other antigens, such as cytokeratins, are also less efficiently demonstrated at this site (15). Alternatively, EBV replication may be down-regulated at the terminal stages of epithelial differentiation. This may have implications for the amount of mature virus shed from OHL.

The replication of EBV in OHL, and by implication probably in normal epithelium, is not typical of herpesviruses. Replication of herpesviruses is usually accompanied by extensive damage to the affected tissue, e.g., ulcers in herpes simplex virus-induced stomatitis or intestinal ulcers caused by cytomegalovirus. In contrast, EBV replication in the epithelial cells of OHL does not lead to ulceration. Although some morphological alterations indicative of cytopathic viral effects, e.g., koilocytosislike changes and nuclear inclusion bodies, are observed (11), virus replication obviously does not lead to cell lysis. Moreover, the epithelial cells in which EBV is replicating in OHL retain their ability to further differentiate, since dysplasia is usually not observed in this lesion. Only the presence of parakeratosis indicates a minor impediment in the normal development of a fully mature epithelium. It seems, therefore, that EBV has evolved to exploit the normal process of epithelial cell differentiation during its replicative cycle, thereby avoiding significant cytopathic effects or associated inflammatory responses.

It has been suggested that the behavior of EBV in OHL resembles that of human papillomavirus infections in which viral latency is maintained in the basal and suprabasal epithelial layers (5, 23). However, in situ hybridization to EBV DNA in OHL failed to detect viral DNA in the lower layers of the lesion. This finding is in agreement with previous studies using biotinylated probes (2, 8). The highly sensitive in situ hybridization technique that we have employed in this study uses ³⁵S-labeled probes and is able to detect the single copy of the EBV genome present in the AW Ramos cell line (14). It may be, therefore, that EBV is not latent in the basal layers of OHL but that the focal areas of

FIG. 4. (a) Demonstration of EBV DNA in an OHL by in situ hybridization using a ^{35}S -labeled DNA probe (3 days of exposure; hematoxylin and eosin counterstaining; $\times 200$). (b) Adjacent section of the same sample stained with MAb BZ1 (APAAP technique; hematoxylin counterstaining; $\times 200$). (c and d) Double-labeling immunohistology and in situ hybridization of an OHL biopsy showing a layer of BZLF1-positive cells underneath a focus of viral replication (c; 3 days of exposure; hematoxylin counterstaining; $\times 100$), which display a weak EBV-specific signal (d; $\times 400$).

FIG. 5. Double immunofluorescence staining of an OHL section for VCA (a; $\times 200$) and BZLF1 (b; $\times 200$), revealing a layer of BZLF1-positive but VCA-negative cells (arrows).

EBV replication represent regions of self-sustained reinfection. If this is the case, our ideas concerning the persistence of EBV in the epithelial compartment may have to be revised. Indeed, other recent reports have questioned the role of epithelium as the permanent reservoir of EBV infection (6, 13, 21). Further studies examining the pattern of EBV latent gene expression in OHL may help resolve this issue.

ACKNOWLEDGMENTS

This work was supported by the Cancer Research Campaign, London, United Kingdom, and by NIH grant PO1 DE07946. Support for G.N. was provided by the Deutsche Forschungsgemeinschaft.

We are indebted to J. R. Harris and A. J. Pinching for access to their patients, to M. Birchall for collecting biopsies, and to H. Stein (Berlin, Germany) for kindly providing the APAAP complex.

REFERENCES

1. Cordell, J. L., B. Falini, W. N. Erber, A. K. Ghosh, Z. Abdulaziz, S. MacDonald, K. A. F. Pulford, H. Stein, and D. Y. Mason. 1984. Immunoenzymatic labelling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). *J. Histochem. Cytochem.* **32**:219-229.
2. De Souza, Y. G., D. Greenspan, J. R. Felton, G. A. Hartzog, M. Hammer, and J. S. Greenspan. 1989. Localization of Epstein-Barr virus DNA in the epithelial cells of oral hairy leukoplakia by *in situ* hybridization on tissue sections. *N. Engl. J. Med.* **320**:1559-1560.
3. Farrell, P. J., D. T. Rowe, C. M. Rooney, and T. Kouzarides. 1989. Epstein Barr virus BZLF1 transactivator specifically binds to a consensus AP-1 site and is related to c-fos. *EMBO J.* **8**:127-132.
4. Friedman-Kien, A. E. 1986. Viral origin of hairy leukoplakia. *Lancet* **ii**:694-695.
5. Gilligan, K., P. Rajadurai, L. Resnick, and N. Raab-Traub. 1990. Epstein-Barr virus small nuclear RNAs are not expressed in permissively infected cells in AIDS-associated leukoplakia. *Proc. Natl. Acad. Sci. USA* **87**:8790-8794.
6. Gratama, J. W., M. A. P. Osterveer, F. E. Zwann, J. Lepoutre, G. Klein, and I. Ernberg. 1988. Eradication of Epstein Barr virus by allogeneic bone marrow transplantation: implications for sites of viral latency. *Proc. Natl. Acad. Sci. USA* **85**:8693-8696.
7. Greenspan, D., J. S. Greenspan, M. Conart, V. Petersen, S. Silverman, and Y. de Souza. 1984. Oral "hairy" leukoplakia in male homosexuals: evidence of association with both papillomavirus and a herpes-group virus. *Lancet* **ii**:831-834.
8. Greenspan, J. S., D. Greenspan, E. Lennette, D. I. Abrams, M. A. Conart, V. Petersen, and U. K. Freese. 1985. Replication of Epstein-Barr virus within the epithelial cells of oral "hairy" leukoplakia, an AIDS-associated lesion. *N. Engl. J. Med.* **313**:1564-1571.
9. Kouzarides, T., G. Packham, A. Cook, and P. J. Farrell. 1991. The BZLF1 protein of Epstein-Barr virus has a coiled-coil dimerisation domain without a heptad leucine repeat but with homology to the C/EBP leucine zipper. *Oncogene* **6**:195-204.
10. Lieberman, P. M., and A. J. Berk. 1990. *In vitro* transcriptional activation, dimerization, and DNA-binding specificity of the Epstein-Barr virus Zta protein. *J. Virol.* **64**:2560-2568.
11. Loning, T., R. P. Henke, R. Reichart, and J. Becker. 1987. *In situ* hybridisation to detect Epstein Barr virus DNA in oral tissues of HIV-infected patients. *Virchows Archiv. A* **412**:127-133.
12. Miller, G. 1990. The switch between latency and replication of Epstein Barr virus. *J. Infect. Dis.* **161**:833-844.
13. Niedobitek, G., S. Hamilton-Dutoit, H. Herbst, T. Finn, M. Vetner, G. Pallesen, and H. Stein. 1989. Identification of Epstein Barr virus-infected cells in tonsils of acute infectious mononucleosis by *in situ* hybridisation. *Hum. Pathol.* **20**:796-799.
14. Niedobitek, G., and H. Herbst. Applications of *in situ* hybridization. *Int. Rev. Exp. Pathol.*, in press.
15. Niedobitek, G., R. Lau, and L. S. Young. Unpublished data.
16. Polack, A., G. Hartl, U. Zimmer, U. K. Freese, G. Laux, K. Takaki, B. Hohn, L. Gissmann, and G. Bornkamm. 1984. A complete set of overlapping cosmid clones of M-ABA virus derived from nasopharyngeal carcinoma and its similarity to other Epstein Barr virus isolates. *Gene* **27**:279-288.
17. Rooney, C. M., D. T. Rowe, T. Ragot, and P. J. Farrell. 1989. The spliced BZLF1 gene of Epstein-Barr virus (EBV) transactivates an early EBV promoter and induces the virus productive cycle. *J. Virol.* **63**:3109-3116.
18. Rooney, C. M., N. Taylor, J. Countryman, H. Jensen, J. Kolman, and G. Miller. 1988. Genome rearrangements activate the Epstein Barr virus gene whose product disrupts latency. *Proc. Natl. Acad. Sci. USA* **85**:9801-9805.
19. Rowe, M., H. S. Evans, L. S. Young, K. Hennessy, E. Kieff, and A. B. Rickinson. 1987. Monoclonal antibodies to the latent membrane protein of Epstein Barr virus reveal heterogeneity of the protein and inducible expression in virus-transformed cells. *J. Gen. Virol.* **68**:1575-1586.
20. Rowe, M., L. S. Young, K. Cadwallader, L. Petti, E. Kieff, and A. B. Rickinson. 1989. Distinction between Epstein-Barr virus type A (EBNA 2A) and type B (EBNA 2B) isolates extends to the EBNA 3 family of nuclear proteins. *J. Virol.* **63**:1031-1039.
21. Yao, Q.-Y., P. Ogan, M. Rowe, M. Wood, and A. B. Rickinson. 1989. Epstein Barr virus-infected B cells persist in the circulation of acyclovir-treated virus carriers. *Int. J. Cancer* **43**:67-71.
22. Young, L. S., C. Alfieri, K. Hennessy, H. Evans, C. O'Hara, C. Anderson, J. Ritz, R. S. Shapiro, A. B. Rickinson, E. Kieff, and J. I. Cohen. 1989. Expression of Epstein-Barr virus transformation associated genes in tissues from patients with EBV lymphoproliferative disease. *N. Engl. J. Med.* **321**:1080-1085.
23. Young, L. S., and J. W. Sixbey. 1988. Epstein Barr virus and epithelial cells: a possible role for the virus in the development of cervical carcinoma. *Cancer Surv.* **7**:507-518.