

## Epstein-Barr virus associated diseases: an update

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### Abstract

The Epstein-Barr virus (EBV), traditionally linked etiologically with infectious mononucleosis (IM), endemic Burkitt lymphoma (BL) and nasopharyngeal carcinoma (NPC) has in recent years been associated with a host of other conditions. Viral strategies for entry into cells and persistence, as well as various molecular mechanisms involved in latency, replication and transformation have been elucidated. EBV termini analysis has demonstrated the essentially clonal nature of BL, NPC and preneoplastic lesions of the nasopharynx. Strain variation between isolates of EBV suggests that differences in epithelial cell tropism among strains may exist. Treatment of EBV-associated syndromes is largely supportive although antivirals may play a role in the management of oral hairy leukoplakia. At the present time, the development of an effective vaccine remains a viable proposition.

**Key words:** Epstein-Barr virus, herpesvirus, infectious mononucleosis, Burkitt lymphoma, nasopharyngeal carcinoma.

### INTRODUCTION

The Epstein-Barr virus (EBV) is a member of the herpesvirus subfamily Gammaherpesviridae.<sup>1</sup> Like other herpes viruses, EBV has a dual cellular tropism. It is a ubiquitous virus that is remarkably successful in evolving an effective strategy for infection, persistence and spread of infection. The virus occurs in nearly 90% of the population, often after an initial asymptomatic subclinical infection.\* Primary infection with EBV causes clinical infectious mononucleosis, a self-limiting lymphoproliferative illness – a disease with a still puzzling predilection for adolescents and young adults.<sup>3,4</sup> EBV has also been etiologically linked to human cancers such as endemic Burkitt's lymphoma, nasopharyngeal carcinoma, and lymphomas in immunocompromised patients.<sup>5</sup> The list of EBV-associated diseases continues to increase.<sup>6-10</sup> Over the last few years, the data accumulated regarding Epstein-Barr virus associated diseases has been voluminous, and this limited review attempts to highlight only key developments in select areas.

#### Infectious mononucleosis

The description and epidemiology of infectious mononucleosis predated by a few decades the recognition of EBV as its cause. Adults, in contrast to children, do not handle EBV infec-

tions well. In Malaysia, as in other developing countries, clinical infectious mononucleosis is rare, and the majority of the population is generally sero-positive to EBV antigens by the age of 10 years." After an acute illness, the virus continues to be shed in the oropharynx, possibly from repository sites in the salivary glandular tissue. This shedding is sustained for months, but gradually lapses; virus can be recovered from the saliva of approximately 20% of healthy carriers, and this figure approaches 90% in post-transplant recipients and immunocompromised hosts, an observation that underscores the importance of the immune response in keeping viral proliferation in check.<sup>12,13</sup> Although 'mononucleosis-like' infections may occur more than once, it is currently accepted that such events are not the result of viral reactivation. Reactivation disease is a phenomenon exclusive to transplant recipients and similarly immunologically deprived individuals.<sup>14</sup> In contrast to other herpes infections where a definite proclivity to recurrences is well established, there has still not been a clearly documented account of symptomatic reactivation EBV disease in a healthy person.

It is clear that the virus can be transmitted by blood transfusion and bone marrow transplantation.<sup>15,16</sup> Viral presence in cervical epithelium and semen has been demonstrated, but there is no proof of sexual transmission." The criteria

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for the diagnosis of **infectious** mononucleosis have been detailed, the strict adherence **which** will identify about 93% of patients with primary EBV infection.<sup>18, 19</sup>

The development of virus-specific antibody tests are useful serological tools, not only for the diagnosis of primary illness but also as adjuncts in **the** evaluation of chronic or malignant **sequelae** of EBV **infection**.<sup>2, 20</sup>

The detection of IgM antibodies to the EBV viral **capsid** antigen (anti-VCA) is presumptive evidence of a recent primary infection. These antibodies evolve quickly with infection, **persist** for weeks to months and do not reappear. Analysis of paired acute and convalescent sera shows a rise, a subsequent fall, and a life-long persistence of IgG VCA, usually in titers ranging from 1:40 to 1:2560. Measurements of IgA anti-VCA is not useful except in the diagnosis and management of nasopharyngeal carcinoma. Antibodies to viral early (pre-DNA synthesis) antigens (anti-EA) of the diffuse or restricted type develop in most primary infections, peak in titers of **less** than 1: 640, and **wane** with time. Although these persist in low titers, they are of no diagnostic significance. **Antibodies** to EBV nuclear antigen (anti-EBNA) are detected relatively late after the onset of symptoms in infectious mononucleosis, so **that** absence in a previously well person who develops acute illness suggest an ongoing EBV infection.

Chronic infectious mononucleosis is a rare, heterogenous and poorly understood syndrome that arises in previously **healthy** men and

women.<sup>21</sup> It **seems** apparent at present that the features of chronic infectious mononucleosis establish it as distinct from the far more **com-**mon chronic fatigue syndrome.

**Molecular biology of Epstein-Barr virus and its mechanisms of B-cell replication**

The EBV genome is a double-stranded G/C rich DNA molecule of approximately 172 kilobases in size. The viral DNA has been cloned **from** many EBV strains and the complete nucleotide **sequence** of one **strain** (B95-8) has been **determined**.<sup>22, 23</sup> **In common** with other herpes viruses, the genome is divided by a number of direct internal repeats into long and **short** unique segments. In addition, the linear genome is flanked by direct terminal repeats of 500 bp in size at **each** end of the linear genome (Fig.1).

There is evidence to support that Epstein-Barr virus infects epithelial cells of the **oropharynx**<sup>24</sup> and the cervix<sup>9</sup> as well as resting B lymphocytes. The receptor for the virus on **the** B lymphocyte is the CD21 molecule, which is **also** the receptor **for** the C3d component of complement.<sup>7</sup> Infection of epithelial cells is permissive, with the release of progeny virion **from** infected cells. In contrast, infection of B lymphocytes usually results in latent infection without replication or release of the virus. CD21 molecules inserted into epithelial cell lines refractory to EBV infection results in these cells being infected by EBV, with expression of latent gene products and **release** of infectious

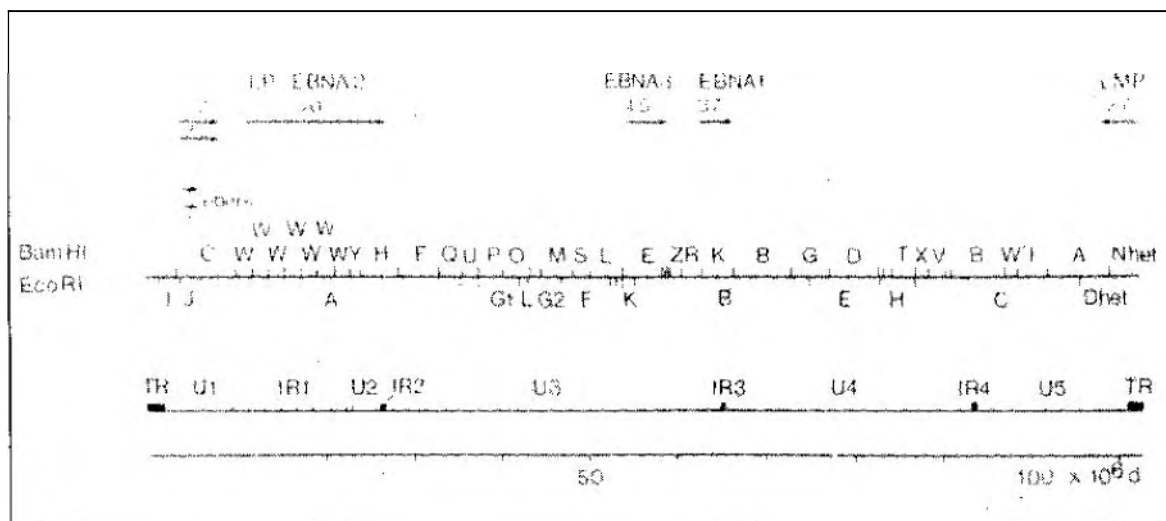


FIG. 1: BamHI and EcoRI restriction map of Epstein-Barr virus. (TR = terminal repeats; IR = internal repeats; U = Unique region).

viral particles.<sup>26</sup> When EBV infects B lymphocytes, its linear genome circularises to form an episome, or extrachromosomal element within the cell nucleus. This results in the transformation of B lymphocytes, which are immortalized, and acquire the ability to proliferate indefinitely.

The heterogeneity in the number of terminal repeats (TR) at either end of the genome between individual EBV virion DNA molecule; has been put to ingenious use in the termini or clonal analysis to assess the status of the EBV viral genome in infected cells.<sup>27</sup> Restriction enzyme digestion of EBV DNA results in the generation of heterogeneously sized restriction fragments, each varying by increments of 500 base pairs, a direct reflection of the number of TR present at either end of the linear genome. The formation of distinctive ladder arrays on gels or Southern blots correlate with "linear virion", "clonal", "latent," or "replicative" patterns. Analysis of the status of EBV termini has confirmed the essentially clonal nature of NPC and Burkitt lymphoma. Curiously, oral hairy leukoplakia (HLP), an exophytic growth of epithelium on the lingual and buccal mucosa of HIV-infected individuals, is almost entirely a replicative EBV infection. anti yields multiple linear arrays on termini analysis, with evidently no episomal forms.<sup>28</sup>

Although EBV remains tightly latent in B lymphocytes, certain chemicals can switch on replicative activity *in vitro*. The expression of EBV BZLF1 gene product, or ZEBRA (Z EBV replication activator) protein, an immediate early gene product, triggers viral replication in latently infected R cells.<sup>29</sup> This protein transactivates the expression of immediate early genes as well as expression of ZEBRA itself. These early genes, in turn, up-regulate the expression of early gene products, which include viral DNA polymerase and thymidine kinase, important for viral replication.<sup>30</sup> Lastly, late gene products are made, which includes structural components of the virion such as VCA and envelope protein gp 350.

### Latency and transformation

The EBV genome is currently believed to encode up to 80 different proteins. Typically, latent infection of R lymphocytes is characterised by the expression of up to eight protein coding genes, and the expression of two small non-coding Pol III transcripts, the EBEB RNA. The protein coding genes are called EBNA

(Epstein-Barr Nuclear Antigen) 1, 2, 3A, 3B, 3C and LP (Leader Protein), latent membrane protein 1 (LMPI) and latent membrane protein 2 (LMP2, or terminal protein [TP]). EBNA-1 is essential in maintaining latency, and is a cis-acting protein which binds to a nucleotide sequence (the oriP) within the BamHI C fragment of the genome. EBNA-1 leads to the transactivation of other EBNA proteins, and EBNA-2 transactivates two EBV latent membrane proteins, LMPI and LMP2. These EBV latent gene products also lead to transactivation of other B-cell genes. EBNA-2 transactivates CD21, CD23 and c-fgr; EBNA-3C transactivates CD21, and LMPI transactivates CD23 and intercellular adhesion molecules ICAM-1, LFA-1 and LFA-3. An autocrine loop is established for the stimulation of EBV-infected B-cells. The truncated and full-length CD23 molecules function as U-cell growth factor and receptor respectively.<sup>31,32</sup> Other growth stimulatory factors include lactic acid and interleukin-6.<sup>32</sup> CD23, which is also a low-affinity receptor for IgE, may be important in antigen presentation in association with major histocompatibility complex (MHC) II antigens and has been shown recently to interact with CD21.<sup>33</sup> c-fgr, a member of the *src* oncogene family, encodes a protein kinase that may be important in the regulation of U-cell growth. Two latent gene products, the EBV-encoded RNAs (EBERs), are the most abundant RNAs expressed *in vitro*: they do not code for proteins, are dispensable for transformation *in vitro*,<sup>34</sup> and may not be necessary for viral replication.<sup>28</sup>

Genetic analysis using viral mutants indicate that EBNA-2 and LMPI are essential for transformation.<sup>35</sup> LMPI acts as a direct oncogene in transformation assays. Expression of LMPI in epithelial cells transforms them morphologically.<sup>36</sup> In R-cell lymphomas, LMPI prevents programmed cell death or apoptosis.<sup>37</sup> LMP2 associates with cellular tyrosine kinase and localises in a cell with LMPI.<sup>38</sup>

At least two EBV types are recognised in human populations; these were formally designated EBV type A and B but since have been redesignated EBV-1 and EBV-2, in line with the nomenclature for HSV. These EBV types differ mainly in the sequences of the viral genes expressed during latent infection and in their ability to transform B lymphocytes. EBV-2 differs from EBV-1 in the BamHI YII region encoding EBV nuclear antigen 2 (EBNA-2).<sup>39</sup> Although earlier studies suggested that EBV-1 virus infection was more prevalent in North

America and Europe, and that **type B** (EBV-2) was more prevalent in Africa, it is now clear that both these strains may be prevalent in the United States" and that persons may indeed be co-infected by both. Recently, a unique variant of EBV has been described in Chinese NPC patients, marked by distinctive restrictive enzyme fragment polymorphisms (KFP), and characterised by loss of a BamHI cut site between W1' and II' fragments at the right end of the genome.<sup>41</sup> A subset of the Chinese strain has an additional BamHI restriction enzyme cut site within the BamHI F fragment at the left end of the genome.<sup>42</sup> A second study also identified a further polymorphism in the Chinese strains, a loss of an XhoI site at the right end of the genome.<sup>43</sup> These data established the fact that the predominant strain associated with NPC in southeast Asia is a variant of EBV type I, marked by BamHI and XhoI polymorphisms. In NPC from areas of low or intermediate incidence, a distinct EBV1 subtype originally identified in the NPC-derived M-ABA strain was detected in approximately 50% of the tumours. It may well be, therefore, that detection of distinct variants in epithelial tumours from areas of varying incidence for a neoplasm may reflect an epithelial cell tropism. Alternatively, it is possible that particular variants of EBV may preferentially establish latent infection in epithelial cells."

#### Lymphocyte responses to Epstein-Barr virus infection

The successful restraint of EBV-infected B lymphocytes is primarily attributable to host T-cell immunity,<sup>45,46</sup> accounting, perhaps, for the rarity of lymphoproliferative disorders of EBV-infected cells. It has become apparent in recent years that B-cells undergo several phenotypic changes that facilitate immortalisation. Little is known about the requirements of growth and survival of EBV-infected cells *in vivo*. During EBV-induced infectious mononucleosis, at least 1 in 10<sup>4</sup> circulating B-cells is infected with EBV,<sup>45</sup> presumably as these cells traverse the mucosa-associated lymphoid tissue in the aerodigestive tract. There has been considerable recent controversy over the derivation of this pool of circulating EBV infected lymphocytes. Moss and colleagues noted that EBV can be detected frequently in the saliva after primary infection, and they proposed that virus in the oropharynx serves as a reservoir to repeatedly infect B cells, which then migrate to the periph-

ery where they are susceptible to killing by cytotoxic T-cells. In this view, the pool of virally affected B-cells would be constantly turning over.<sup>47</sup>

An alternative model, now more widely accepted, is that the EBV-infected cells are long-lived, and are perhaps the very cells that were originally infected or their progeny.<sup>48</sup> In support of the latter model, acyclovir therapy has been shown to eliminate, or at least reduce, EBV shedding in the saliva, although it has no effect on the number of circulating cells infected with EBV.<sup>49</sup> Moreover, EBNA-typing of EBV-infected cells during a 3-year period in a patient who had undergone ablative therapy and subsequent bone marrow transplantation showed them to be donor derived." During the 3 years, no B cells that carried the recipient's previous viral genotype could be found. Thus, EBV-infected cells can be long-lived, and *de novo* infection with virus intermittently released in the oropharynx and elsewhere may be uncommon after primary infection. Recently, EBV-specific secretory IgA mediated mechanisms have been proposed as a third possible route for viral entry into mucosal epithelial cells.<sup>51</sup>

#### T-cell regulation of Epstein-Barr virus infection

T-cell derived immunity in EBV-exposed individuals comprise of both an activated cytotoxic T-cell response (neither EBV specific nor HLA-restricted), and specific cytotoxic T-cell reactions." Recent studies have shown extensive homology in the predicted amino acid sequences of murine interleukin-10, human interleukin 10 and the protein product BCRF-1 (viral interleukin-10)<sup>50</sup>; BCRF-1 is an open-reading frame in the EBV genome known to be expressed during viral replication. Functionally, human and viral interleukin-10 share several properties, including inhibition of gamma-interferon secretion and suppression of the T-cell proliferation in response to antigen and mitogen.<sup>52</sup> By inhibiting T-cell growth and gamma-interferon production, viral interleukin-10 may limit host responses directed at eliminating the virus.

The virus specificity of cytotoxic T cell clones derived from EBV-seropositive persons has been mapped. The clones recognised EBNA-2, EBNA-3A, EBNA-3C, and LMP1,<sup>53-55</sup> but not EBNA-1. The lack of detectable EBNA-1-specific T-cell clones may have important implications regarding the cellular immune contain-

ment of EBV-infected cells. Epstein-Barr virus nuclear antigen is required to maintain the viral episome in B cells, so its expression is obligatory; however, the expression of other genes is not required. The failure of EBV to express certain genes, such as LMP and EBNA-2, *in vivo* may allow B cells to avoid cytotoxic T-cell recognition and clearance.

### Lymphoproliferation and neoplasia

Studies have begun to clarify the molecular properties of several EBV-associated lymphoproliferative and neoplastic diseases, as well as the role of the virus in their development. Burkitt lymphoma is a monoclonal cell tumour typically containing an 8/14 or 8/22 translocation that ends in a dysregulation of the endogenous *c-myc* oncogene. In Africa, more than 90% of Burkitt lymphomas contain EBV genomes, whereas only 20% of non-endemic Burkitt do the same. Cells in Burkitt lymphoma express EBERs and EBNA-1 only. The chromosomal translocation in Burkitt lymphoma may supplant the need for expression of additional EBV latent, growth-transforming genes. Further EBNA-2, EBNA-3A, EBNA-3C and LMP1 are targets for the destruction of EBV-infected B-cells by cytotoxic T-cells.<sup>56</sup> Thus the expression of these genes would enhance the immune clearance of cells; their down-regulation would facilitate survival. Many EBV-associated lymphoproliferative disorders are recognised in patients with congenital and acquired cellular immune deficiency. X-linked lymphoproliferative disease is a rare disorder of young boys who develop fulminant mononucleosis after EBV infection. Over two-thirds of patients die of haemorrhage and 100% die by the age of 40 years. EBV associated lymphoproliferative disease also occurs in 1% and 3% of bone marrow, kidney or liver transplant recipients. About 5% and 10% of heart and heart-lung transplants have developed EBV-associated B-cell tumours.<sup>57</sup> The marked immunosuppression in these patients may allow the outgrowth of EBV-infected cells. The reversal of these lesions after the reduction of immunosuppressive therapy emphasises the role of the immune system in controlling EBV infection in the normal host. In transplant recipients, as well as in patients with AIDS, EBV-associated B-cell tumours may have either polyclonal, monoclonal or mixed phenotypes. About one-third of B-cell lymphomas that develop in patient with AIDS contain EBV genomes.<sup>57</sup> Most of the latent gene

products are also expressed by the tumour cells. Death from progressive expansion of tumours, immunodeficiency and opportunistic infection is frequent. Patients with Hodgkin's disease often have elevated levels of antibody to EBV antigens before or at the time of presentation with the disease. Recently, tissues from 20-40% of patients with Hodgkin's disease have been found to contain EBV genomes, usually within Reed-Sternberg cells.<sup>8</sup> Most of these cases associated with EBV are of the more aggressive, nodular sclerosing or mixed cellularity subtypes.<sup>58</sup> These tumours also express LMP-1, but not EBNA-2.

### Nasopharyngeal carcinoma

Nasopharyngeal carcinoma (NPC) is an epithelial malignancy with a global distribution although there are characteristic geographical and population differences in incidence.<sup>59</sup> The disease is endemic in persons from southern China and in southeast Asia where the estimated incidence may be as much as 20% of all cancer cases. The strain variation of EBV isolates from areas of varying incidence of NPC has been remarked upon earlier. Elevation of serum IgA EBV VCA is typical and highly diagnostic of the disease, often predating the development of NPC by several years. These data suggest that EBV reactivation and replication occurs before the onset of NPC. In the clinical setting, the absolute levels of elevation of IgA titers correlate well with tumour bulk and tumour recurrence. Three histological types of nasopharyngeal carcinoma are recognised (designated as WHO 1, 2 and 3). The less differentiated forms of NPC (WHO types 2 and 3) have been consistently shown to be EBV-related; the relationship of EBV with Type 1 NPC is controversial, and conflicting views have been published in the literature.<sup>60,61</sup> Tissues from patients with the disease show expression of EBNA-1, the EBERs, and LMP1 and LMP2 genes. We recently discovered a previously unidentified open reading frame at the 3' end of the genome arising from with BamHI A, termed BARFO in specimens of NPC (Fig.2).<sup>62</sup> Sequence analysis showed that the initial AUG initiation codon could initiate translation of a 20 kDa polypeptide, and the *in vitro* translation product of the BARFO cDNA was precipitable with sera from patients with NPC, suggesting that the ORF is expressed *in vivo*.<sup>63</sup> Other studies have shown that the expression of BARFO is consistent in both EBV-associated epithelial<sup>62</sup> as well

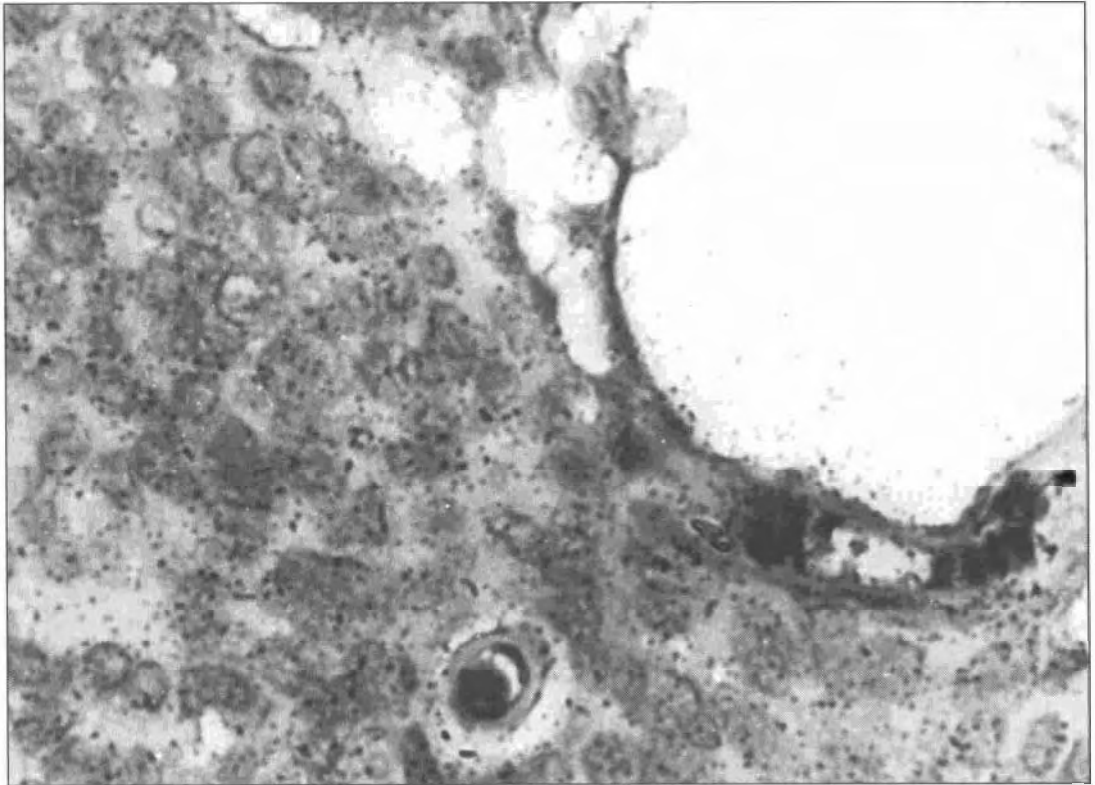


FIG. 2: C15, mouse skin. *In-situ* hybridization of SP<sup>35</sup>-labelled antisense riboprobe to BamHI A. C15 is a nude-mouse passaged NPC cell line. Dark grains (indicating presence of BARFO transcripts) are present uniformly in all tumour cells. Normal fat cells and hair follicles of mouse skin do not hybridize with the probe. X 200.

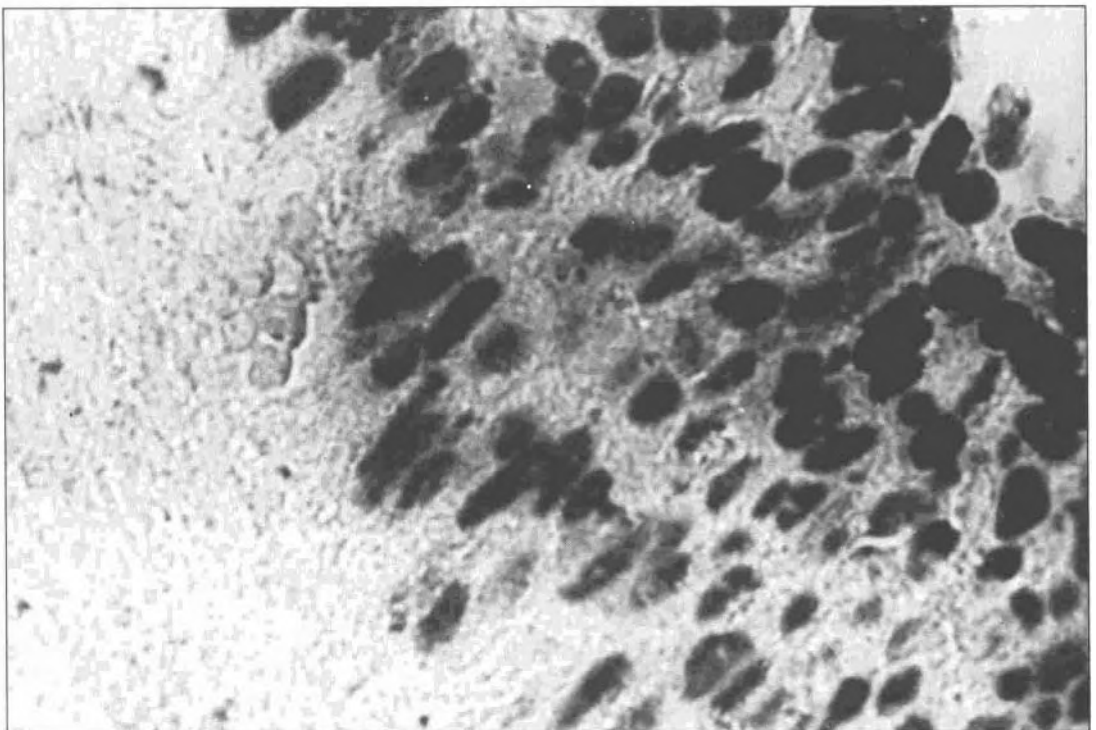


FIG. 3: Focus of carcinoma *in-situ* in the nasopharynx. *In-situ* hybridization with a digoxigenin-labelled EBER1 probe shows strong positivity. X 300.





