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Chapter 11

Microbiological Diagnosis of Infection by the Epstein-Barr Virus: Pathogenic Basis

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Abstract

This study is a review of current knowledge concerning the pathogeny of infection by the Epstein-Barr virus. An analysis is made of the biological events that take place in primary infection, persistent chronic infection, oncogenic transformation, and viral co-infections. Phenomena surrounding the non-specific clonal stimulation of B lymphocytes are explained. A review is also made of clinical laboratory tests available for the serodiagnosis of primary and persistent chronic infections, including the clinical significance of different classes of antibody, the value of IgG avidity assays and the use of recombinant antigens in diagnostic equipment. The characteristics of commercially available equipment are also reported. Laboratory procedures based on previous protocols are proposed. Finally, the tests that can be used to detect virus-related tumors are discussed.

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Introduction

The *Herpesviridae* family includes a large number of viruses pathogenic to animals and humans. Amongst these latter, various subfamilies and species have been described (Table 1). These include the subfamily α-*Herpesvirinae*, containing the simple herpesvirus strains 1 and 2 and the varicella-zoster virus, the subfamily β-*Herpesvirinae*, containing the human herpesvirus strains 6 and 7 and the human cytomegalovirus and the subfamily γ-*Herpesvirinae*, containing human herpesvirus 8 and the Epstein-Barr virus. Unlike the rest of human pathogenic viruses, herpes viruses, after initial infection, whether symptomatic or not, remain in the individual's system for the rest of his life, coming to form part of his microbiota. This persistence occurs in the form of slight or latent infections, with or without later cell immortalization (transformation). Latent infections, which are reactivated with greater or lesser frequency throughout life, are a characteristic feature of herpes viruses.

Table 1. Classification of the *Herpesviridae* family

α-Herpesvirinae		*β-Herpesvirinae*		*γ-Herpesvirinae*	
Simplexvirus (VHS)	*Varicellovirus* (VVZ)	*Roseolovirus* (VHH-6 y 7)	*Cytomegalovirus* (CMV)	*Rhadinovirus* (VHH-8)	*Lymphocryptovirus* (VEB)

We intend to focus here upon the Epstein-Barr virus (EBV), or human herpesvirus 4. More than 90% of the world's adult population is infected with this virus, 70% of them having caught it before reaching the age of 30. Recently a series of advances have been made in our knowledge and technological ability that would advise a thorough revision and the establishment of pathogenic bases for the microbiological diagnosis of EBV infection, such as:

a) The impact that new technology has had on laboratory testing.
b) A greater knowledge of the pathogeny of EBV infection, which would lead to a better understanding of the problems attached to the serodiagnosis of infections caused by EBV, cytomegalovirus and human herpesvirus 6.
c) Their relationship with newly discovered tumoral processes.
d) The growing frequency of immunocompromised patients (those with transplants or neoplasms, or suffering from HIV, for example), in whom EBV infection can manifest itself in different ways.
e) The availability of new antiviral drugs.

In this chapter we shall be reviewing different microbiological tests based upon the pathogeny of the infection.

Structure of EBV

EBV, like all herpes viruses, has a lipoprotein envelope containing glycoproteins in its structure, which derive from the nuclear membrane or Golgi apparatus of the infected cell and protrude outwards from the envelope (Fig. 1). Between the icosahedral capsid and the envelope there is a zone of proteins known as the tegument. The genome consists of a single molecule of linear double–stranded DNA (186-Kb) within the viral particles, and episomal (circular, non-integrated) DNA within the nucleus of the infected cells. The viral DNA consists of one long section (UL) and a short one (US), with internal repeat zones (IRs) flanked by terminal repeat zones (TRs) (Fig. 2). In the infected cells the TR ends of the genome join together to form the episome. Regions U3 and U5 in the UL section allow the virus to follow a lytic cycle. Latency and later reactivation, on the other hand, are due to the oriP zone in region U1 of the US section [1].

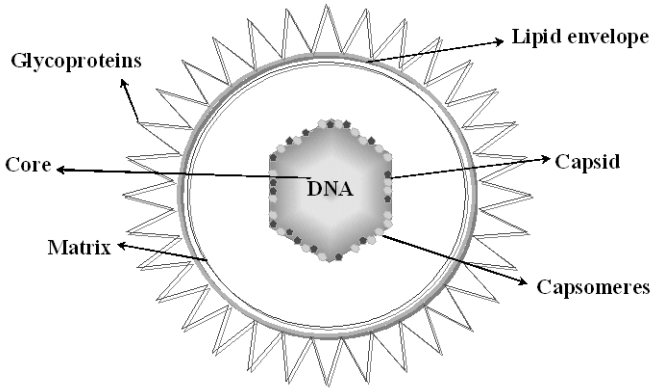


Figure 1. Structure of the Epstein-Barr virus

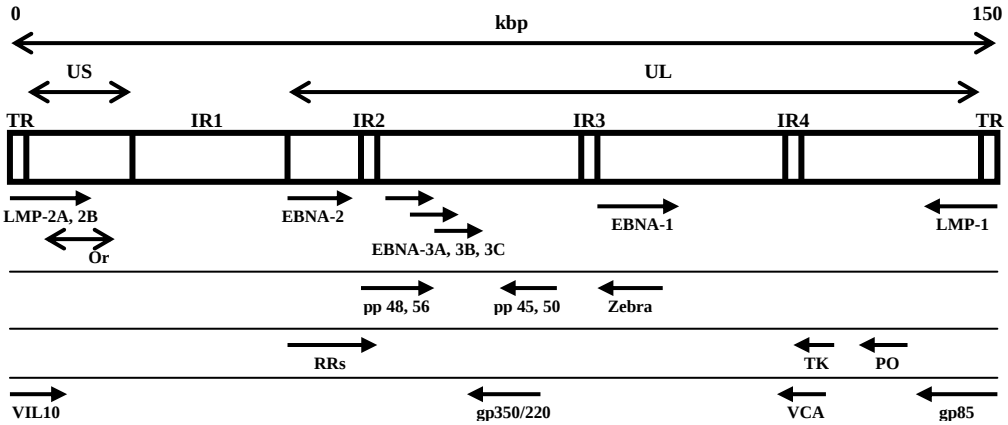


Figure 2. Viral DNA and protein synthesis

Replicative Cycle and EBV Antigens

The virus penetrates the cell wall (Fig. 3) and migrates to the nucleus, where replication occurs. The genome is transcribed in three different phases, each of which depends upon reading the genes in the previous phase [2-4].

The initial, or immediate phase begins after the entry of the virus and leads to the synthesis of primary or regulatory proteins for the subsequent phases, which are necessary for the later synthesis of nucleic acids and structural proteins. They are located in the nucleus of the cell, are antigenic and are present throughout the viral cycle. They are known, among other names, as ZEBRA proteins and are encoded by the EBV-BZLF-1 open reading frame.

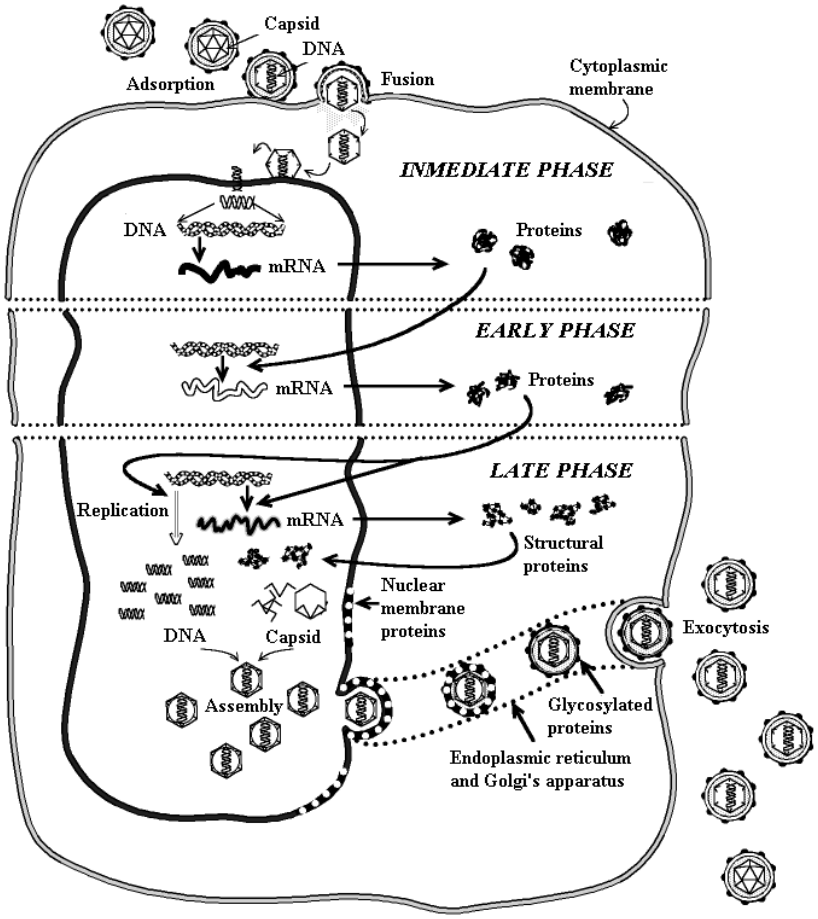


Figure 3. Replicative EBV phases

In the second, or early phase, early and late-early genes are transcribed, giving rise to specific proteins such as phosphorylases and polymerase DNA for the synthesis of viral DNA. Antigen proteins are also synthesised. These regulate the subsequent phases and are located initially in the cell membrane (early-EMA membrane antigen) and diffused

throughout the cytoplasm (early diffuse-EA/D antigen), later to become restricted to the nucleus (early restricted-EA/R antigen).

During the third, late phase, most of the late genes of the virus are transcribed and structural proteins are synthesised such as those of the capsid [capsid antigens (VCAs), a very specific one of 18 kDa and others of 40 and 135 kDa], and those of the envelope [late membrane antigens (LMAs)] of 85, 110 and 220/350 kDa. Capsids, which then incorporate the DNA, and glycoproteins, located initially in the nuclear membrane and Golgi's apparatus, are generated. The capsids approach the glycoproteins to create new complete particles.

Once formed, the virus leaves the cell, first emerging slowly through the Golgi apparatus and then lysing the cell due to its massive exit. Empty extracellular capsids and protein macromolecules, all of them antigenic, are frequently present during this process. These phases are successive and may coexist in the same cell.

In some cases cell infection may be latent (macromolecules are generated but no viral synthesis takes place); the genome is not integrated into the cell nucleus and replication is forestalled. This latent phase is characterised by an absence (or only very small presence) of any acute symptoms of infection and a dearth of antigens. During this phase infection is not completely inactive but at least 9 mRNA polyadenylated and two small non-polyadenylated RNAs (EBERs) are transcribed. Six of the mRNAs encode nuclear antigens (EBNAs 1-6) whilst the other three translate into latent membrane proteins (LMPs 1, 2A and 2B). An analysis of the latent proteins in cell cultures has revealed the existence of three different types of latency (I, II and III) (cf. Table 2).

- Type I has been observed in biopsies and cell lines of group-I Burkitt's lymphoma as well as in infected circulating lymphocytes in healthy individuals. In this type, EBNA 1, EBER RNAs and RNAs of the *Bam-HI* region of EBV are expressed selectively.
- Type II has been observed in nasal-pharyngeal carcinoma tumours, although it has also been reproduced in fibroblast hybrids and epithelial lines that express the receptor CD21, and also in Hodgkin's lymphoma. It expresses selectively EBNA 1, LMP 1 and LMP 2 in an irregular fashion. EBER RNAs and RNAs of the *BAM-HI* region are also expressed. Unlike Type I, its pattern of expression is less well understood.
- Type III has been observed in lymphoblastoid cell lines, in post-transplant lymphoproliferative processes and cell lines of group-III Burkitt's lymphoma. It is characterised by the expression of EBNAs 1-6, LMPs 1 and 2, EBER and *BAM-H1* region.

Table 2. Latency patterns of EBV

	Latency		
	I	II	III
EBER	+	+	+
EBNA1	+	+	+
EBNA2	-	-	+
LMP1	-	+	+
LMP2	-	-	+

One of the EBV proteins that shows oncogenic capacity is LMP 1. It has 386 amino acids and forms an integral part of the membrane. It contains six transmembrane domains and a long carboxyl-terminal cytoplasmic domain. The presence of LMP 1 changes the morphology of both B and epithelial cells. It reduces the response in the latter to normal differentiation signals and increases their invasive potential within the collagen matrix. This oncogenic capacity has been proved in *in vitro* studies of cell cultures and its presence has been seen in epithelial tissue biopsies from the nasal-pharyngeal cavity [5-9]. It has also been reported to be capable of inducing CaM-kinase and adhesion molecules LFA 1, LFA 3, ICAM 1 and CD23 in human B lymphocytes. Finally, LMP 1 mimics the TNFR superfamily receptors p80TNFR, CD40 and CD30 by using their intracellular signalling factors TRAF and TRAD. Via their interaction with these factors and by mechanisms not yet fully understood LMP 1 triggers two phosphorylation pathways that lead to the activation of transcription factors NF-κB and AP1.

EBNA is one of the few genes that are essential to the immortalization of B lymphocytes, behaving as they do as a transcriptional activator of viral and cellular genes. It is a phosphorylated polypeptide antigen containing 487 amino acids. It increases the transcription of the cell activation markers CD21 and CD23 and also the tyrosine kinase c-fgr. CD23 is a growth factor for B cells and c-fgr is a kinase protein that induces the multiplication of lymphocyte B. Furthermore, EBNA 2 can transactivate the promoters of the viral genes of LMP 1 and 2 and the promoter of *Bam HI-C*, which is required for the transcription of the nuclear antigens.

Two genotypes of the virus, EBV 1 and 2, have been described. EBV 1 is prevalent throughout the world and is more capable than EBV 2 of transforming B lymphocytes into an established cell line (immortalization). EBV 2 is found more frequently in central Africa. These strains can be distinguished by variations in EBNA 2 and EBNA 3A and C. An analysis of EBER and LMP 1 also allows us to differentiate between these viral strains [10].

Apart from primary infections and reactivations, it has been shown that it is possible for an individual to be infected by a different strain of the same virus that he is already harbouring in latent form. This phenomenon is known as reinfection but its clinical repercussions are not yet well understood due to the fact that when the immunological response fails, reactivation and reinfection are both possible. In this respect, because of their relatively low immune response, pregnant women may suffer reactivation of herpes viruses, which are not usually associated with the grave effects to the foetus that a primary infection

might incurr. In any of these three circumstances, primary infection, reactivation or reinfection, clinical symptoms may or may not be apparent.

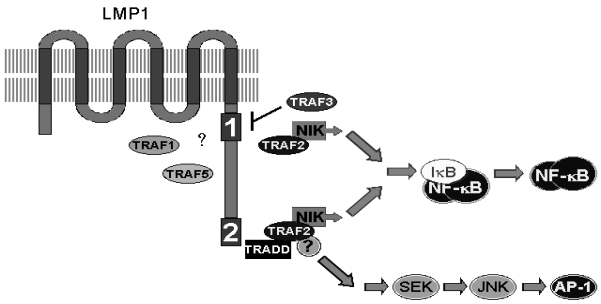


Figure 4. Mechanism for the activity of LMP 1

Pathogeny of the Infections

Primary Infection

Entry

The virus is generally transmitted by direct oral contact (saliva), after which it colonises the cells of the nasal-pharyngeal epithelium, where it establishes a lythic replication cycle. It is possible, but much less frequent, for the virus to be transmitted percutaneously, by sexual contact or through transplanted organs. In both the latter cases direct infection of the white blood cells also occurs [10].

The virus binds to the cells by the interaction of the 350/220 kDa glycoprotein in its envelope with the CD21 receptor of the epithelial cells and B lymphocytes. The number of cell receptors increases concomitantly with the level of infection, thus facilitating over-infection. Furthermore, with the production of new virions adjacent cells are also infected. CD21 is a membrane receptor that associates with cell proteins CD19 and p53, both of which participate in controlling the normal cell cycle. EBV establishes itself in the cell within cytoplasmic vesicles (in the case of primary B lymphocytes), from whence it releases its nucleocapsid into the cytoplasm. This is then transported to the nucleus, where it is degraded.

During this phase EBV infects the B lymphocytes, either preceded or not by initial replication in the nasal-pharyngeal epithelium, and proliferates in peripheral blood and lymph nodes. Thus, local adenopathy and secondary viraemia ensue, accompanied by unspecific symptoms.

Viraemia

Viraemia occurs both freely and in the B- and T-helper lymphocytes. It leads to a specific immunological response by the T-helper, natural killer and T-cytotoxic lymphocytes. Furthermore, the B lymphocytes, stimulated by the monocytes, generate specific plasmatic cells against the virus, whilst the B lymphocytes infected by EBV are also stimulated and transformed into distinct clones of the plasmatic cells. These latter, while they remain alive

and functionally stimulated, synthesise antibodies against any antigen, initially of the IgM class, and if the cell dies (lythic cycle) or the stimulus does not persist (latency or immortalization), specific IgG is not then synthesised.

This important and distinct immunological stimulation is followed by a downmodulation of the response which is mediated by regulatory T cells. These cells are large, very abundant and functionally very active in EBV infection. They are atypical lymphocytes that resemble monocytes. The presence of these cells together with swelling of the lymph nodes, liver and spleen is characteristic of the mononucleosis syndrome, and hence the name "infectious mononucleosis" (IM) given to the main illness caused by EBV (given that it is not monocytosis).

The infection of the T-helper lymphocytes and activation of the regulatory T lymphocytes induces a temporary immunosuppression with variable biological repercussions. Thus, individuals with a previously compromised cellular function encounter difficulty in combating EBV infections, which may lead to the development of a chronic or immunoproliferative illness. Furthermore, if the individual already harbours a herpes-based illness he may undergo a reactivation of this original complaint during infection with EBV [11 – 13].

Finally, during this phase of viraemia the clinical symptoms can vary from unspecific to those proper to infectious mononucleosis.

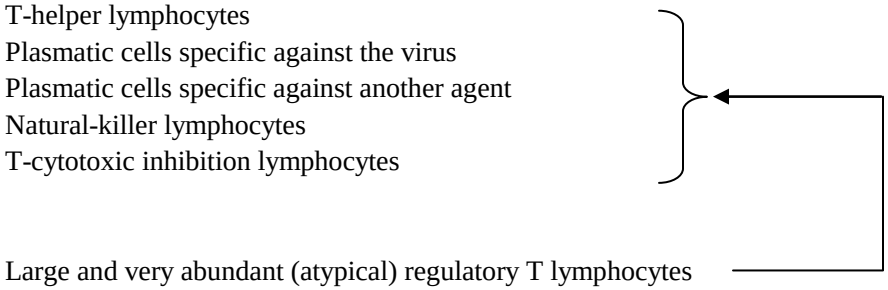


Figure 5. Immunological activation after primary infection by EBV

Latency and Focalization

Immunity reduces viral replication to a phase of latency and focalization, which is normally lifelong. This occurs mainly in the B lymphocytes, although the basal cells of the nasal-pharyngeal epithelium, some T lymphocytes and possibly the epithelium of the uterine cervix may also harbour latent forms of the virus. During the latency phase the number of infected B lymphocytes remains at around 10^5.

While the virus persists surface antigens are scarcely expressed, which hinders the activation of the immune response. Furthermore, together with those described for LMP-1, the virus inhibits the apoptosis mechanisms in the infected lymphocytes due to a viral protein encoded by the gene *BHFR1*. Lastly, the proteins encoded by the genes *BCRF1* and *BARF1* inhibit the synthesis of interferons. In this way EBV can guarantee its own survival and that of the infected cells [14-23].

In some cases immunity is not sufficient to control the infection and this gives rise to a persistent, chronic replication in various organs or anatomical structures of the body. In a few patients the cells infected by EBV also proliferate, causing lymphoproliferative and neoplastic epithelial processes. For this reason this latent phase may be followed by persistent replication or a neoplastic transformation of the infected cell. The development of these events depends very much upon the condition of the immunological system of the individual in question [24]. This of course does not rule out the possibility that in some cases such cofactors as geographical location, for example, may contribute to the appearance of neoplasms either by offering the infected cells an additional proliferative advantage or by weakening the immune response against them. And in all this, genetic predisposition may also have a part to play.

Reactivation

Reactivation of the virus in the form of a lythic cycle and subsequent cell destruction have been described as occurring after the latent or chronic infection phases. This is believed to be associated with a lowering of the immune response in moments of stress, systemic infection or injury, which may lead to a recurrence of EBV infection, with or without any specific clinical symptoms. Such recurrence implies damage to the organ in which the virus is seated together with secondary viraemia. At the same time alterations to the immune system caused by EBV infection may reactivate other previous infections [25].

Finally, it is worth noting that due to its latency the infected individual becomes a carrier of the virus and can spread it within his environment due to later reactivations.

Clinical Processes

Primary Infection

Primary infection usually occurs during infancy and is generally asymptomatic. When it occurs during adolescence it manifests itself in 50% of cases in the form of an IM syndrome. In general terms, the older the individual the more likely he is to suffer symptoms of the infection. The incubation period ranges between one and three weeks. Primary infection by EBV is the most frequent cause of IM in adolescents (90%), but nevertheless, other microorganisms and factors, such as cytomegalovirus (the most frequent cause of mononucleosis in those over 25), adenovirus, human herpesvirus strains 6 and 7, the parotiditis and rubeola viruses and *Toxoplasma gondii* are also known to be involved. The IM syndrome caused by EBV may sometimes not be diagnosed because it mimics infections of the upper respiratory tract, leukaemia or Hodgkin's disease. It does not follow any clearly defined seasonal rhythm although it does seem to appear more frequently in winter and spring. Unlike other human herpes viruses, EBV contagion within the family is uncommon. In most cases patients show fever, pharyngitis or tonsillitis and firm (though not hardened), painful adenopathies Less frequently there is palpebral oedema, stiffness of the neck,

erythema, mid-palate petechiae and swollen liver and spleen. A haemogram shows 70% lymphocytosis and nuclear hyperbasophylia. The most severe complication can be rupture of the spleen and thrombocytopenia.. Neurological, pulmonary and cardiac complications may occur but are less common.

In some cases the primary infection does not cure itself and remains active for months or even years. In this case there may be splenomegaly, pancytopenia, an inversion of the CD4/CD8 lymphocyte ratio, hypergammaglobulinemia and a reduction in the number of natural-killer lymphocytes. In other cases the latency that follows primary infection is interrupted by clinical reoccurrences such as the reactivation of an endogenous virus or, less frequently, by reinfections by the same or another strain of herpesvirus. In immunocompromised individuals serious illnesses often ensue, causing damage to any bodily organ. Amongst such illnesses are Guillain-Barré's syndrome, uveitis, oral furry leukaplakia, hepatitis and interstitial lymphocytic pneumonitis.

Neoplastic Processes

Our understanding of the relationship between EBV and human pathology began with the description by Dennis Burkitt in 1962 of an endemic lymphoma in children in equatorial Africa and the subsequent identification by Epstein and Barr of a new virus present in lymphoma cells. This virus has turned out to be the one most consistently associated with malign cell-transformation processes driven by proteins present during the latency phase, which are capable of interfering with apoptosis. The latency proteins, expressed under favourable conditions, trigger the oncogenic transformation of B lymphocytes (Burkitt's lymphoma) and epithelial cells (nasal-pharyngeal carcinoma). What is more, the detection of sequences of the EBV genome in other tumours suggests that it might be involved both in the pathogenesis of immunocompetent hosts, where it is associated with Hodgkin's lymphoma, other lymphomas, thymic lymphoepithelioma and cancer of the salivary glands, and also in immunocompromised hosts, where it is associated with immunoblastic lymphomas in individuals with AIDS and lymphoproliferative illnesses in transplant patients [4, 22, 26, 27].

Direct Microbiological Diagnosis

Due to the high prevalence of EBV infection, to demonstrate the presence of the illness clinical samples and tests must be used that are not influenced by mere colonisation, as occurs with the serodiagnosis of the illness when not due to primary infection. In such cases direct diagnostic techniques are preferred.

Clinical Samples

These depend upon the location of the illness. Alginate swabs should be avoided in cultures as they might damage the infected cell, and likewise heparin in blood samples, which reduces the infectivity of the viral particle.

Samples may be taken from spinal fluid, the biopsies of tissues or viscera, vitreous humour, saliva, secretion from the tonsils, the nasal-pharyngeal cavity, from the uterine cervix or from the lower respiratory tract (although cytomegalovirus is often encountered in bronchoalveolar washes from infected individuals and thus it is difficult to evaluate their significance unless typical cell alterations are also present). Due to the fact that the virus is often only located intermittently or locally, to increase the efficiency of the study various samples must be analysed; in general three should be enough.

Complete blood samples may be studied (the least frequent option), or just some of its isolated components such as plasma, the leukoplakia layer, or the lymphocytes, all of which are potentially toxic for the culture cells. Given that this virus may form part of the normal microbiota, studies of blood samples help towards a greater level of clinical significance but are never definitive.

With transplant patients the state of the infection should be controlled using blood and saliva samples once a week for the time that immunosuppression lasts.

Finally, the samples may require a means of transport, which is usually the same maintenance medium as that of the viral culture, with antibiotics. Samples should be kept at 4ºC for up to 48 h or at -20ºC for greater lengths of time. Nevertheless, a delay in making the culture will always reduce its reliability.

Cell Culture

This is possibly the most specific method, allowing as it does the retrieval of the virus from any sample, such as B lymphocytes, and a semi-quantitative measurement of the infectious virions. This procedure is complicated, time-consuming and not very cost effective, rendering it impractical on a daily basis. It is less reliable with reactivations and latent infections compared to forms of primary infection and does not distinguish between one or another form unless the viral load is quantified.

This technique can be carried out in a tube or on microtiter plates. A lymphoid cell line is used (with culture controls of up to 60 days) or cord B lymphocytes (with culture controls of up to 15 days), with phytohaemaglutinin or anti-CD3 antibodies and a maintenance culture medium containing interleukin-2, which facilitates cell infection.

Visualizing the Virus

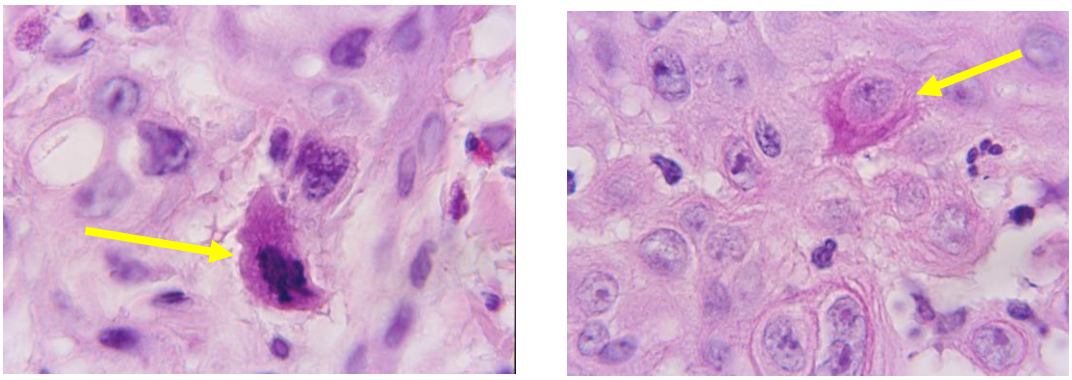
Studies include optical microscopy and various forms of electron microscopy. The latter techniques detect the complete virions developed in the lythic infection and are extremely effective in diagnostic terms, but their complexity makes them suitable only for reference centres. Optical microscopy, when used to study infected culture media, allows the technician to observe, refringent, giant and lymphoblastoid multinuclear cells in proliferation, and also intranuclear and cytoplasmic inclusions.

Immunofluorescent or immunoperoxidase tests on clinical samples of potentially infected cultures are efficient in diagnosing infection. These assays can detect late proteins in the

lythic cycle (VCA and EA/D) in oral furry leukaplakia lesions and post-transplant hepatitis, and latency proteins (mainly EBNA-1, LMP-1 and LMP-2) in associated neoplastic processes (Fig. 6). In various studies into samples of squamous cells from oral carcinomas [28-32] we have found that:

1. Almost 20% of the tumours contained EBV DNA, mainly those located around the side of the tongue.
2. LMP-1 was present in 86% of these cases.
3. EBV DNA was associated with a lower level of cell differentiation, worse clinical prognosis and an absence of cytoplasmic p53.

The expression of p53 was not a prognostic marker



A **B**

Figure 6. Expression of LMP-1 in neoplastic cells from cytoplasmic (A) and membrane (B)

Early regulatory proteins such as ZEBRAs are reliable indicators of the early presence of the virus in culture.

In general terms, small quantities of the antigens are expressed in the cells and it is necessary to have recourse to anti-complement immunofluorescence and a very careful preparation and conservation of the sample to demonstrate the presence of the virus. Overall the results show high sensitivity and specificity in the detection of illness caused by EBV. A quantitative analysis allows an evaluation of the clinical response to treatment, but technical false negatives must be allowed for. Thus, when antigens are detected via immunoperoxides the destructive phase of the endogenous peridoxase might eliminate the virus's presence.

Molecular Diagnosis

As far as this aspect of diagnosis is concerned, two points must be emphasised: viral genome can be detected in the lymphocytes of infected patients showing no signs of illness

and also in neoplasms associated with the virus. We shall now look into the clinical significance of each of the laboratory tests for arriving at a molecular diagnosis [1, 33-36].

In Situ Hybridization

This is based on the detection of EBER and is the most specific and important assay for defining viral infection in tissue. It constitutes the reference test for detecting latent infection in tissues embedded in paraffin and cytological preparations. Nevertheless, the degradation of RNA may produce false negative results. EBER is present in tumours associated with the virus and thus its detection can be used to show clinical recurrences of the tumour. In practice, however, the detection of EBV DNA is not used very much except in situations in which the RNA is degraded, in which case a biotinylated probe for the *Bam H1* "W" fragment of EBV is used.

Southern Blotting

This assay is used to discover the degree of clonality of the structure of the viral genome in the infected tissues. Terminal repeat sequences at the end of each linear DNA molecule are analysed. In this way patterns of bands are obtained which may be monoclonal, oligoclonal or polyclonal. In practice it has been found that tumours associated with monoclonal patterns have the worst prognosis.

Amplification of Nucleic Acid

DNA can be detected either quantitatively or qualitatively. In the former case the positive predictive value to define the illness is one of 50% and the negative value 100%, and the disappearance of the symptoms is not always associated with a negative test result. The low predictive value is based upon the presence of EBV DNA in the lymphocytes of healthy individuals, for which reason the qualitative detection of the virus does not distinguish between colonisation (chronic infection) and reactivation. This poor behaviour improves, however, with samples of spinal fluid, serum and plasma. Thus in the spinal fluid of AIDS patients a positive result indicates primary lymphoma of the central nervous system and does not require confirmation by cerebral biopsy. A negative result in this sample after treatment would be associated with a satisfactory reduction of the tumour. Studies of serum and plasma would only detect viral genome in currently ill patients or those with a high systemic EBV load.

To increase the diagnostic reliability of the qualitative studies either the mRNA is studied or viral DNA is quantified; a positive result is associated with a greater level of replication. Thus the mRNA of EBNA-1, LMP-2 and BARF-1 has been investigated using the NASBA method. The mRNA of EBNA-1 appears in all the lymphomas associated with EBV, that of LMP-2 in Hodgkin's lymphomas and nasal-pharyngeal carcinoma, and finally, that of BARF-1 seems also to be related to nasal-pharyngeal carcinoma.

A knowledge of the viral load in real time in spinal fluid, blood (total, cells, serum or plasma), saliva and so on is of great value in the evaluation of patients who run the risk of clinical recurrence or developing an EBV-associated neoplasm. When this test is applied a cut-off point with an optimum predictive value together with a record of the clinical

parameters and complementary laboratory techniques should be used in the interpretation of the results. Thus in transplant patients changes in the viral load can be seen when the associated illness appears, which return to normal via specific treatment.

Finally, it is worth noting that molecular studies may be positive in paediatric transplant patients because of a primary infection and that the RFLP test is useful for genotyping clinical isolates.

Indirect Microbiological Diagnosis

Infectious mononucleosis, the main manifestation of EBV infection, can be diagnosed in serum samples. Cell culture techniques, because of their difficulty and the time involved, are scarcely ever applied in this clinical context. Its clinical manifestations range from the benign to those with grave implications for the health of the patient. These latter manifestations of the illness are those that provide most reason for serodiagnosis, with the caveat, however, that a large part of the adult population will present antibodies revealing a primary infection earlier in their lives and a present latent infection and that these antibodies will vary according to the age of the individual and the functionality of his immune system.

Serodiagnosis also helps before a transplant to identify those patients with a high risk of developing a lymphoproliferative syndrome, such as children, recipients of intestine or lungs, patients who receive high doses of immunosuppressors and those who might undergo rejection of a transplanted organ. This risk is especially grave when there is a primary EBV infection, and hence the importance of locating seronegative subjects. Patients testing seronegative to cytomegalovirus should also be identified because a primary infection by this virus is a risk factor in the reactivation of EBV.

Assays Currently Available to Detect the Different Antibody Isotypes

Specific antibodies against EBV of the IgG, IgA and IgM classes can be detected by ELISA, latex (less sensitive than ELISA), indirect immunofluorescence (IF), anti-complementary IF (a variant of IF used to detect antiEBNA antibodies because of its greater sensitivity), Western blotting and complement fixing (of low sensitivity for seroprevalence studies).

For the IF assay and its variants several types of antigen can be used:

1. The established cell line P3HR-1 stimulated with phorbol ester and fixed in acetone. This line derives from B lymphocytes from Burkitt's lymphoma and contains VCA.
2. The Raji line (non-producer of virions), which contains EBNA.
3. The Raji line stimulated by overinfection with EBV or treated with tumour-inducing agents (iododeoxyuridine or sodium butyrate), which contains EA. To select the EA/D or EA/R variants their susceptibility to methanol and acetone are examined, EA/D being resistant to methanol and EA/R to acetone; EBNA is resistant to both.

All these studies should be accompanied by tests using non-infected B lymphocytes (i.e. the established cell line BJAB) to exclude any possibility that the positive result might be due to the presence of anti-nuclear antibodies. Cells with plasmids that express the different EBNA-2s are used to distinguish between infection by strains 1 and 2 of the virus. At present IF can only be recommended to check doubtful ELISA results or as a research tool.

For ELISA assays, extracts of infected cells (which express EBNA, EMA, EA VCA and free virus), viral proteins purified by chromatography (gp 125 of the VCA complex), recombinant proteins and synthetic peptides (EBNA-1 p72 and p58; VCA p18 and p150; EA/D p54) can all be used. Most available ELISAs that detect IgM are indirect and only a few use immunocapture methods. ELISAs that use cell extracts as antigens are more sensitive than those which do not, whilst at the same time being less specific. Nowadays ELISAs that do not use cell extracts as antigens provide comparable results to those given by IF. In short, it is advisable in practice to use an ELISA assay with antigen extract backed up by IF to confirm any doubtful results.

The greatest difficulty in evaluating the results of serological studies is the absence of any reference test in the microbiological diagnosis of the state of infection such a cell culture would provide, because of the difficulty in carrying it out. Other problems are presented by the possibility of new aetiological agents of IM, which might give atypical serological patterns, and a lack of standards for serodiagnosis. This problem has in part been resolved by the publication of the *Medical Research Council Research Standard A, 66/235 for infectious mononucleosis serum*, obtainable from the WHO National Institute for Biological Standards and Controls (UK).

Primary infection is characterised by the presence of IgM, IgA and IgG. Past infections do not show IgA or IgM but IgG is present. Reactivations and reinfections by another herpes strain (more frequent in immunocompromised patients) are associated with either positive or negative IgA and IgM, and IgG is mobilised. In primary infections IgA can be detected by IF 10 days after the appearance of symptoms and it persists for 2 to 3 months. Studies are complicated when various IgMs against different infectious agents, which may or may nor be related genetically, coexist. This may be due to simultaneous or successive viral infections, the use in the diagnostic kits of poorly characterized antigens that may contain common epitopes, persistence of the infection, unspecific clonal stimulation of the B lymphocytes or a reactivation of the latent virus with associated viraemia. On the other hand, it may be that no IgMs are present, as is commonly the case in immunocompromised individuals. Lastly, IgMs may have a molecular weight of either 7S or 19S, the former being those detected in non-primary infections. An attempt has been made to use these differences between IgMs to distinguish between those that are generated in primary infections and those involved in the rest of cases, such as reinfections and reactivations. Laboratory results of detected IgM are usually expressed qualitatively.

IgGs can be detected by IF after 15 days' infection and remain in low quantities for years. IgG studies can tell of susceptibility to a primary infection, if it is not present, or to a potential reactivation during immunosuppression, if it is present. It appears early on in primary infection due to the significant viraemia detected in this early stage of the infection and the fact that high-sensitivity assays such as ELISA are used. Thus, from a practical point of view, it is almost impossible to demonstrate seroconversion in primary infection; its levels only

increase during recurrencies that cause a significant systemic effect. IgG can be present for a long time, sometimes for life. This may be a consequence of latency, reinfection or reactivation and thus its detection is useful when making a selection of seropositive samples in a blood bank, in seroepidemiological studies or the diagnosis of a previous infection. Laboratory results of IgG studies are expressed either qualitatively, semi-quantitatively (by titers) or quantitatively (due to the availability of the standards mentioned above, which allow the results to be expressed in international units). The concept of titer is commonly used to demonstrate the seroconversion of antibodies between two blood samples, but it should be born in mind that this increase does not always have any clinical significance. One such example is the increase that may be found in anti-EBV IgG during cytomegalovirus infections which do not add up to a clinical recurrence. Furthermore, anamnesic reactions that cause an apparent increase in the antibody titre may occur.

The affinity or "avidity" of IgG to neutralize the antigen increases progressively throughout the primary infection. In this way the quantity of IgG with less avidity falls concomitantly with an increase in IgG with a normal avidity for neutralizing the antigen. Six months after the onset of the primary infection practically all the IgG present shows significant avidity. Thus if the greater part of all the IgG present in a blood sample is of low avidity, this will point to a recent (less than six months) primary EBV infection. Once a sufficient degree of avidity has been reached it remains the same throughout the individual's life regardless of any changes of levels in serum, as a consequence for example of reinfections or reactivations. Thus, whenever IgG proves positive, the measurement of its avidity is a result that should be routinely included in laboratory reports to help decide whether, instead of being a primary infection, it may be a significant clinical recurrence or a non-specific clonal stimulus. This factor is particularly important in the presence of strong viraemia. In many cases the clinical symptoms are not very specific, the IgG response is early and seroconversion cannot be reliably shown, but an analysis of its avidity can be made, either alongside various IgMs, or even with just one if more studies have not been made. Furthermore, when there is sufficient clinical evidence to suspect EBV infection but IgM proves negative, an assay to discover the avidity of IgG will confirm or rule out whether this negative is false or not. Thus processes of very different clinical importance can be compared.

IgA can also be studied. This antibody behaves very similarly to IgM although it occurs in lesser quantities and is present for a shorter time than the latter. Neither are commercial kits sensitive enough to detect it. Its presence in serum is associated with persistent chronic EBV infection but the difficulties involved in detecting its presence, false negatives due to an excess of IgG and false positives in individuals with chronic liver disease have all contributed to its being hardly studied in hospital laboratories.

Finally, false negatives and irregularity in the evolution of the antibodies are not infrequent in children and immunocompromised patients. In cases of meningitis and encephalitis they appear quite late in spinal fluid and thus are of little use as far as early diagnosis is concerned, their value being restricted to the confirmation of direct diagnosis. And lastly, when they are present they have to be shown to have developed locally in response to the infection [37-47].

Antibodies Synthesised During Infection

To understand the nature of the antibodies synthesised during the course of infection by EBV it is important to remember that antigens are expressed during the replicative cycle of the virus and the pathogeny of the primary infection.

Heterophilic Antibodies (Paul Bunnel-Davidson Test)

These are anti-ovine or -bovine IgMs which can be detected in plasma six days after the onset of symptoms and persist until three months afterwards. They may also be present in cases of hepatitis A and HIV, and also with arthritis and lymphoid neoplasms, due to the non-specific stimulation of B lymphocytes. Thus heterophilic antigens cannot be put down to EBV-specific antigens.

They are usually determined in purified erythrocytes by latex or immunochromatographic assays with no need for any previous absorption. In practice, when investigated within a correct clinical framework, the specificity of these assays is almost 100% and their sensitivity around 90%, when the population studied does not include children younger than five, due to the fact that false negatives have been found in 50% of very young children and 10% - 20% of ill adults. The fact that this illness is not very frequent in these groups of the population maintains the general validity of the detection of these antibodies.

In cases of IM with negative heterophilic antibodies the diagnostic method is one of serological assays to detect specific antibodies against EBV, just as it is with patients presenting heterophilic antibodies but no typical clinical symptoms. These antibodies do not appear in infections caused by other herpes viruses and thus they help to distinguish between cases of mononucleosis by different viruses. The quantity of these antibodies bears no relation to the seriousness of the illness. Finally, the greatest usefulness of this test lies in the fact that if mononucleosis is suspected it can be carried out in real time.

Anti-VCA Antibodies

Anti-VCA IgM appears initially, although this may be the consequence of a primary infection by cytomegalovirus, which causes a polyclonal stimulus. In up to 30% of cases of mononucleosis by EBV, on the other hand, specific IgM against cytomegalovirus is detectable. IgM may be absent during the primary infection or it may persist for months or even years afterwards. Anti-VCA IgM is followed by the early synthesis of IgG but high levels of this latter antibody do not indicate recent infection however high its level might be. Anti-VCA IgM usually disappears during convalescence whilst IgG is detectable for life.

Anti-EA IgG

Some pathologists consider that the detection of this antibody is the most reliable sign of primary infection, although it may be that only antibodies against EA/R are to be found in

young children. The quantity has also been related by some pathologists to the seriousness of the illness. Before the appearance of anti EA/D antibodies, anti-VCA IgMs and anti-EBNA-2 (the latter by ELISA) and heterophilic antibodies are detectable. Lastly, a response to the early antigens may persist in 4% - 20% of people who have suffered the infection, and thus they cannot be considered as being a definite marker of a recent infection. Anti-EA/D IgG disappears during convalescence.

Anti-EBNA Antibodies

Although the EBNA proteins are the first to be expressed in infected cells (mainly EBNA-2 and then EBNA-1), the antibodies against these proteins cannot be detected by IF until some months later, usually at the same time that anti-VCA IgM decreases, but they then remain in the body for life. This delay in detection can be put down to a combination of factors: the lower sensitivity of IF and the fact that this technique only detects anti-EBNA-1 IgG, and the antigen's lack of exposure to the immune system until the infected cell disintegrates. On the other hand, if ELISA is used to detect anti-EBNA-2 IgM, this behaves in a similar way to anti-VCA IgM. Thus, in overall terms, EBNA-2 antibodies are detected before EBNA-1. In this way, the detection of anti-EBNA-2 via ELISA provides the first marker of recent primary infection. Finally, it is quite possible for the immune reaction to the EBNA-1 antigen, studied by IF, never to appear or to turn out negative in the case of immunocompromised individuals.

The Behaviour of Antibodies in EBV-Related Illness

Primary infection

On the basis of the evolution of the synthesis of the antibodies, in possible cases of IM (Fig. 7) an assay for heterophilic antibodies is advisable in patients of whatever age. This test is specific enough if positive to confirm a diagnosis of mononucleosis caused by EBV with no need of further assays to detect anti-EBV antibodies. If it gives a negative result further assays are required to detect anti-VCA IgG, anti-VCA IgM and anti-EBNA IgG antibodies, always bearing in mind that immunocompromised patients may well present atypical patterns. To help differentiate between these difficult serodiagnostic situations, an association of anti-VCA and anti-EA IgG together with an absence of anti EBNA-1 IgG, detected by IF, indicate acute EBV infection. Past infection is indicated by the presence of anti-VCA and anti EBNA-1 IgG together with an absence of heterophilic antibodies and anti-VCA IgM. Finally, it must be emphasised that a diagnosis of primary infection should not be arrived at on the sole evidence of a positive anti-VCA IgM because this could easily be a false result. In the case of a positive IgM the avidity of the IgGs present should be studied, which should on the whole turn out to be of scarce affinity towards the antigen. Due to the complexity of serodiagnosis different procedural protocols can be proposed, one of which might be that set out in Table 3 [37 – 47].

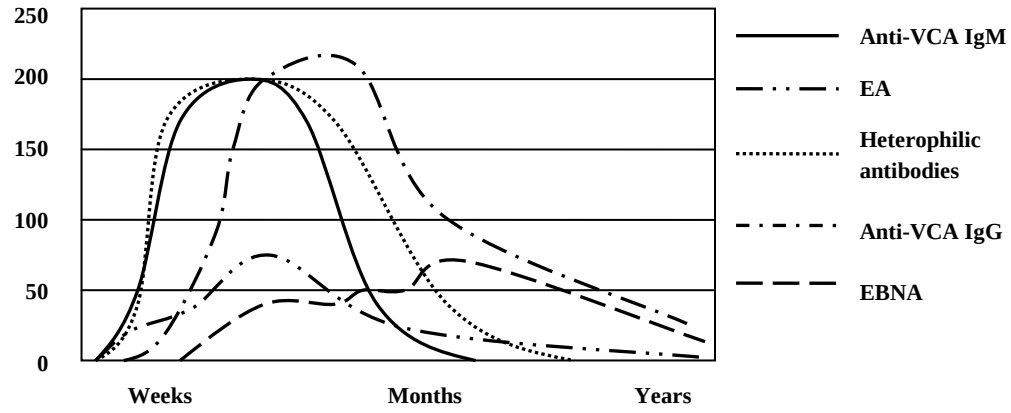


Figure 7. Evolution in infectious mononucleosis of heterophilic (HA) and specific antibodies as observed by immunofluorescence

Table 3. Procedural protocol to discover the state of EBV infection by applying an ELISA assay to an antigenic extract of infected cells expressing all the antigens of the virus's replication cycle

IgG < 25 IU/ml	IgM	(+)	Recent Acute Infection		
		(-)	No Infection		
IgG ≥ 25 IU/ml	IgM	(+)	Low Avidity IgG	≥ 55%	Recent Primary Infection
				< 55%	a) Primary Infection (> 3 months)
					b) Clonal Activity of B Lymphocytes
		(-)	Past Infection		

Illness Associated with Chronic Infection

In illness associated with chronic infection lymphocytosis does not exist and the antibodies are present to varying degrees. Furthermore, as the anti-EBNA antibodies are lymphocyte-T dependent, if anti-EBNA antibodies are absent, the situation could be mistaken for a primary infection when in fact it is a reactivation, reinfection or a latent state of the virus.

The evolution of the state of the antibodies, mainly detected by ELISA, in illness associated with chronic EBV infection is shown in Table 4. This serological behaviour must always be qualified by the fact that with immunocompromised patients the interpretation of serological results is complex and difficult due to the scarce or nil production of antibodies by these individuals. Thus, serological diagnosis is not always a valid alternative with these patients.

In Burkitt's lymphoma, nasal-pharyngeal carcinoma and immunocompromised patients there are normally high titers of antibodies against VCA and EA. The anti EA response is directed against EA/R in Burkitt's lymphoma and immunocompromised patients and against

EA/D in nasal-pharyngeal sufferers. Most patients with nasal-pharyngeal carcinoma develop an IgA response against different EBV proteins, mainly VCA and EA. Anti-VCA IgA has a sensitivity of almost 100% for detecting the illness and anti-EA IgA a specificity also of about 100%. Thus a combination of these assays can prove valuable in the detection and follow up of the illness (stable and falling values indicate a better prognosis) [45-47].

Table 4. Serological markers in EBV infection

	Heterophilic antibodies	Anti-VCA antibodies			Anti-EA antibodies		Anti-EBNA
		IgG	IgM	IgA	Diffuse	Restricted	
Infectious mononucleosis	+	+++	++++	++	+	±	-
Past infection	±	++	-	-	-	-	+
Active chronic infection	-	++++	++	+++	+++	++	±
Lymphoproliferative processes	-	+	+	-	-	-	-
Burkitt's lymphoma	-	+++	-	-	±	++	+
Nasal-pharyngeal carcinoma	-	++++	-	++++	++++	++	++++

In summary, as can be seen throughout this chapter, despite great advances in the diagnosis of acute EBV infection and processes related to its chronic state, there is still a lot to be achieved in the field.

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