The Biology of Circulating B Lymphocytes Infected with Epstein-Barr Virus During Infectious Mononucleosis

JAMES E. ROBINSON, M.D.

Department of Pediatrics and Epidemiology and Public Health, Yale University School of Medicine, New Haven, Connecticut

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EBV-infected B cells are present in blood during acute infectious mononucleosis (IM) in surprisingly large numbers. These cells share with cells transformed by EBV in vitro the capacity for unlimited proliferation, but this capacity is quite restricted in the normal host. The unusual extent of plasmacytic differentiation seen in infected cells in vivo early in IM and the loss of this differentiation in the late stages of the disease suggest that these cells are subject to the same immunoregulatory mechanisms that affect normal B cells. The host response to virus-altered B cells is quite complex but is perhaps best viewed as an in vivo autologous mixed lymphocyte reaction initiated by the interaction of T cells with virus-activated B cells.

Infectious mononucleosis (IM) is a usually benign lymphoproliferative disease that results from primary infection with Epstein-Barr virus (EBV). This virus, a member of the herpes virus group, is unique among human pathogens in its tropism for B lymphocytes and its capacity to induce the continuous proliferation of infected cells in vitro [1]. In addition, the virus stimulates the synthesis and secretion of immunoglobulins by cells infected in vitro; for this reason it is frequently referred to as a polyclonal activator of B cells [2]. In spite of the transforming capacity of EBV, the major manifestation of lymphoproliferation in peripheral blood during IM is the presence of large numbers of atypical lymphocytes, a majority of which have T lymphocyte characteristics [3]. Presumably, these cells are immunologically stimulated in response to the disease. It should be emphasized that the lymphoproliferative process in IM is not confined to blood, as lymphocytic infiltration can occur in lymph nodes and in virtually any organ or tissue.

Much attention has been focused on various aspects of humoral and cellular immune responses in IM, while studies on how EBV interacts with the cells it infects in vivo have been relatively few, perhaps because of the difficulties involved in studying these cells. However, it seems reasonable to presume that EBV-infected cells are the initiators of the complex cascade of immunologic events that account for many of the manifestations of the disease. Thus, knowledge of these cells— their number, proliferative capacity, and stage of differentiation—is essential for a clear understanding of the pathogenesis of IM.

This review is not intended to be a comprehensive discussion of the pathogenesis of IM but rather to summarize what has been learned about EBV-infected cells in
the peripheral blood of mononucleosis patients. Much of what is discussed here is based on results obtained from studies in our laboratory. Where it seems appropriate, this information is interpreted in light of what is known about some aspects of the immune response during IM.

**PRESENCE AND NUMBER OF CIRCULATING EBV-INFECTED LYMPHOCYTES IN IM**

That EBV-infected lymphocytes were present in the blood of patients during IM was originally inferred from the ready outgrowth of EBV-transformed cell lines from cultured blood lymphocytes [4]. After primary EBV infection a lifelong carrier state develops in which small numbers of latently infected cells are present in blood and lymphoid tissues. Early estimates of the number of infected cells in the circulation were based on a quantitative culture assay in microtiter plates in which the minimum number of blood lymphocytes needed to give rise to cell lines was determined [5]. According to these estimates, as many as two in 10,000 circulating mononuclear cells carried the virus during acute disease. This number decreased during the course of the disease and during convalescence a minimum of 10^7 cells was required to establish cell lines.

The demonstration that EBV nuclear antigen (EBNA) was expressed by infected peripheral blood lymphocytes during IM [6,7] provided a more direct means to study cells infected *in vivo*. In order to detect small numbers of EBNA-positive cells, T cells must first be removed from peripheral blood lymphocytes [6]. Thus, T cell-depleted lymphocytes have been used in subsequent studies involving blood from normal IM patients. The number of antigen-positive cells in the circulation depends on the duration of clinical symptoms. By the end of the first week of illness, about 6–20 percent of this population of cells are EBNA-positive [8]. The number of antigen-positive cells declines rapidly in the second week of illness and reaches low levels (0.1–0.6 percent) during the third week. We have been unable to detect EBNA after this time, although Katsuki et al. [7] have detected very small numbers of EBNA-positive cells in IM blood taken as long as 4–8 weeks after onset.

These results show that there is an almost alarming number of circulating infected cells late in the first week of IM. Since it is not possible to obtain blood specimens earlier in the disease, it is not possible to know if even higher proportions of antigen-positive cells are present before a patient develops significant symptoms.

The decline in the number of EBNA-positive cells in blood during the course of IM is most likely brought about through the action of cytotoxic T cells. Early studies suggested that T cells with specific cytotoxicity for EBV-transformed blood are present in IM blood [9]. Further studies suggested that this cytotoxicity was not restricted to HLA-compatible targets [10]. However, the specificity of these cells for EBV-related targets is now being questioned. Recent evidence points to there being several populations of T cells, which, when activated *in vitro* by mitogens or in mixed lymphocyte cultures or *in vivo* during IM, are equally capable of killing EBV-related and unrelated target cells [11]. Regardless of their specificity, these cytotoxic cells probably play an important role in the elimination of EBV-infected cells.

Following IM or any primary EBV infection, long-term T-cell immunity with specificity for EBV-transformed cells does develop [12]. The demonstration of cells responsible for this immunity depends on an *in vitro* sensitization step involving exposure of memory T cells to autologous EBV-transformed cells in order to generate effector T cells. The cytotoxicity that develops appears to be specific for EBV-
transformed cells and is HLA-restricted; i.e., requires HLA-compatible targets [12]. It is thought that such memory T cells are responsible for the control of latently infected cells.

The antigen recognized by secondary cytotoxic T cells appears de novo on the cell surface of B cells soon after infection. Whether it is a virus gene product or a unique altered cellular antigen has not been determined. It has not as yet been serologically defined.

**PROLIFERATIVE CAPACITY OF EBV-INFECTED CELLS IN VIVO**

The capacity of EBV to transform lymphocytes in vitro, together with the presence of the virus in two human malignancies, Burkitt lymphoma and nasopharyngeal carcinoma, raises the question of whether the cells infected in vivo during IM are actually transformed. Seeming to support this hypothesis was the outgrowth of cell lines from cultured blood obtained during and after mononucleosis. However, Rickinson et al. [13] found that cell lines from IM blood do not necessarily represent progeny of cells infected in vivo. These investigators showed that a majority of LCL growing from co-cultures of IM blood and human umbilical cord blood did not arise from the patient's cells but were of fetal origin. Since the cord blood lymphocyte population did not contain endogenous EBV, transfer of virus from one population of cells to another had to occur to explain the results. In addition, human serum containing EBV antibody markedly reduced the incidence of spontaneous transformation.

Two conclusions from these observations were obvious: the transfer of infected cells from the in vivo environment to in vitro conditions activates productive viral replication in at least some of the genome-positive cells; an unknown proportion of transformed cells that grow out from cultures of IM blood actually become infected and transformed in vitro and do not represent progeny of circulating virus-carrying cells. These results suggested to Rickinson et al. [13] that EBV genome-positive cells in IM blood are only latently infected and that their growth properties were not affected. However, the induction of lytic viral replication in explanted cells does not exclude the possibility that EBV induces proliferation in the B cells that it infects in vivo, since virus production can occur in established transformed cell lines [14].

There are now several lines of evidence that support the concept that EBV causes a primary B-cell proliferation in IM. The first is that colonies of EBNA-positive cells can be grown from IM blood lymphocytes plated in semisolid medium [7]. Since the conditions of this method of culture prevent the transfer of virus from cell to cell, this finding suggests that at least some of the genome-positive cells are capable of direct proliferation into transformed cell lines. One objection that can be raised here is that clumps of cells could be plated together, allowing for the transfer of virus to uninfected cells.

A second line of evidence supporting the hypothesis that EBV infection enhances growth potential of B cells in vivo comes from studies in our laboratory in which we demonstrated that some EBNA-positive cells could undergo mitosis while in the circulation or shortly after being placed in culture [8]. Taking advantage of the physical association of EBV with metaphase chromosomes [15], we incubated B lymphocytes, freshly isolated from IM patients in the first week of illness, with colcemid for two hours to arrest dividing cells in metaphase. Smears of these cells were then stained for EBNA. In cells treated with colcemid immediately after the cells were separated, only about one in 200 EBNA cells were in mitosis. If the B cells were
cultured overnight (about 18 hours) at 37°C before exposing them to colcemid, a burst of mitotic activity in the antigen-positive population occurred; 3–5 percent of the cells with EBNA were in mitosis. These results are direct evidence that circulating virus-carrying cells in acute IM have the potential to divide in vivo. However, this proliferative potential appears to be restricted. Removal of the B cells from the host resulted in increased mitotic activity, suggesting that the cells had been released from the influence of an inhibitory environment.

The third line of evidence that EBV infection of B cells causes proliferation in vivo comes from studies of patients who develop fatal lymphoproliferative syndromes during primary EBV infection. A number of such patients have been reported—some of them appear to have a genetic susceptibility which is X-linked, whereas others occur sporadically in individuals of either sex and affect individuals of different ages [16]. The relationship of the lymphoproliferation in these patients to EBV was firmly established when we studied a four-year-old girl who developed a poorly differentiated lymphoplasmacytic malignancy during primary EBV infection that presented initially as infectious mononucleosis [17]. The tumor cells and the majority of peripheral blood lymphocytes were EBNA-positive and contained multiple copies of the EBV genome. The tumor was polyclonal in origin as direct cloning of the cells in semisolid medium gave rise to cell lines that produced lambda and kappa light chains. A number of patients with similar polyclonal B-cell tumors in which EBNA or EBV-DNA is found have subsequently been reported. In every case, including the one we studied, an underlying immune defect was either strongly suspected or known to be present. The critical immune mechanisms that operate in normal individuals to restrict the proliferation of EBV-transformed cells in vivo, but that are absent in patients in whom unchecked proliferation occurs, have not yet been identified. Presumably cytotoxic and suppressor T cells, whether specific or nonspecific, and humoral factors, such as antibodies, interferons, and lymphokines, acting individually or in combination, are involved in these control mechanisms.

DIFFERENTIATION OF EBNA-POSITIVE CELLS IN IM

In the child who developed malignancy during IM, the tumor cells morphologically resembled plasma cells. Indeed, most of them contained not only EBNA but also cytoplasmic immunoglobulins (cIg) detectable by direct immunofluorescence [17]. It was somewhat surprising to find that 45 percent of the peripheral blood lymphocytes synthesized cIgA, 15 percent cIgG, and only 2 percent cIgM. To determine whether the degree of differentiation and the distribution of immunoglobulin heavy chains in this patient was extraordinary, and, therefore, might suggest an unusual host cell-virus interaction, we undertook a study of patients with uncomplicated IM. Using two-color immunofluorescence which allowed the simultaneous identification of EBNA-positive cells and the demonstration of cytoplasmic immunoglobulins, we found that in the first week of illness 70–80 percent of the EBNA-positive cells also contained cytoplasmic immunoglobulins and thus showed plasmacytic differentiation. Heavy chain expression in these cells in six IM patients was similar to that seen in the patient with fatal disease; i.e., EBNA-positive cells with cIgA and cIgG were more common than were cells with cIgM [18].

As noted above, the number of EBNA-positive cells in IM blood decreased rapidly in the second and third weeks of illness. This decrease was accompanied by a loss of differentiation, as cIg was detected in a low proportion of the remaining EBNA-positive cells. Cytoplasmic immunoglobulin synthesis may be inhibited in these in-
fected cells by the suppressor T cells which have been shown to appear in the blood of IM patients in the second week of illness [19,20]. These cells inhibit immunoglobulin secretion in B cells polyclonally activated in vitro by pokeweed mitogen or by EBV and thus are not specific for EBV-infected cells [19,20]. It has been proposed [20] that suppressor cells play a role in the depression of cell-mediated immunity that characteristically occurs in IM [21].

EBV infection of B cells in itself is sufficient to cause some differentiation of B cells [2], but preliminary observations in our laboratory show that cytoplasmic immunoglobulin synthesis occurs in fewer cells transformed in vitro than it does in vivo during early IM. This suggests that circulating infected cells are subject to immunoregulation not only by suppressor but also by helper mechanisms. Helper factors that polyclonally activate B cells in vitro are known to be produced during autologous mixed lymphocyte reactions [22]. Since EBV-transformed cells are very potent stimulators of autologous T cells [23], it is reasonable to view the initial interaction of host T cells with virus-altered B cells in terms of an in vivo mixed lymphocyte reaction which sets in motion the first wave of reactive lymphoproliferation. This initial activation of T cells could stimulate B-cell differentiation as well as account for the presence of nonspecifically cytotoxic T cells as has been proposed by Klein et al. [11].

Patients with IM characteristically develop hypergammaglobulinemia and produce a number of unusual antibodies. These include Paul-Bunnell heterophil antibodies, the presence of which is diagnostic of IM, and a number of autoreactive antibodies. It has been widely assumed that B cells, polyclonally activated by EBV infection in vivo, are the source of these antibodies [20]; however, our results do not support this notion. The unusual antibodies in IM are almost always of the IgM class. By contrast, most EBNA-positive cells produced IgG and IgA and relatively few produced IgM. In addition, in some IM patients increased numbers of uninfected plasmacytic cells were present [18], suggesting that normal B cells are also activated in IM. This leaves open the possibility that uninfected, as well as infected, B cells may be the source of the unusual antibodies that are produced in IM.

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