Case Report

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Atypical CD3+ CD4^{low} Cell Population in a Boy with Fatal EBV-Infection

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Abstract

A previously healthy 10-year-old Greek boy born to nonconsanguineous healthy parents developed progressive liver disease after acute infectious mononucleosis. EBV-induced autoimmune hepatitis was suspected and treatment was started with high-dose prednisolone, acyclovir and intravenous immunoglobulins. Despite therapy, his liver function continuously deteriorated and the child died 9 months later in profound immune deficiency from candida septicemia. Flow cytometric analysis of his lymphocytes revealed a major subpopulation of atypical cells (20.3%) which were CD3+, fitted into the lymphocyte gate but showed a very low level of CD4 expression, comparable to that of monocytes. After short-time cell culture, the cells became adherent and developed granules and dendrites. We conclude that these cells may represent strongly activated CD4+ T lymphocytes with downregulated CD4 expression or a subtype of dendritic cells.

Introduction

Infectious mononucleosis is a common self-limiting disease of children and young adults caused by Epstein-Barr virus (EBV). The main clinical and laboratory features are fever, lymphadenopathy and pharyngitis. In about 50% of patients, hepatosplenomegaly occurs with elevated transaminases and occasionally jaundice [1, 2]. Acute infectious mononucleosis is accompanied by an absolute lymphocytosis, usually with more than 20% atypical lymphocytes. Most of these are monoclonal or oligoclonal activated CD8+ cells specific for EBV antigens [3]. Neutropenia, especially in young children, is relatively common [4, 5]. In patients with different types of congenital, acquired or iatrogenic immune deficiency, the response to EBV infection may be inadequate resulting in uncontrolled proliferation of virus-containing cells [6–9]. Liver disease is present in almost all affected cases, causing death in about half of the patients [10]. Since these fatal EBV infections in previously healthy children are rare, very few data exist concerning lymphocyte subpopulations in the peripheral blood [11, 12]. We describe a 10-year-old boy with fatal EBV-associated infectious mononucleosis, who showed a population of atypical CD3+ CD4^{low} but CD8^{-} cells in his peripheral blood.

Case Report

A previously healthy 10-year-old boy born to nonconsanguineous Greek parents developed a morbiliform exanthema, fever, jaundice and hepatosplenomegaly, without enlarged lymph nodes, 10 days after treatment with amoxicillin. At that time EBV IgG ELISA titres were extremely high (92,000 U/l). Three months after disease onset, liver function tests were still abnormal. Acute infectious hepatitis (hepatitis A, hepatitis B and hepatitis C virus, human immunodeficiency virus, leptospirosis, herpes simplex virus, cytomegalovirus, EBV IgM were negative) and metabolic diseases (α_1-antitrypsin deficiency, Wilson's disease) were excluded and the boy was transferred for liver biopsy. Laboratory tests revealed elevated liver enzymes

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(alanine aminotransferase 417 U/l, aspartate aminotransferase 304 U/l) and conjugated hyperbilirubinemia (6.4 mg/dl), signs of hemolysis, elevated IgG (22 g/l) and low positive titers (1:40) of antinuclear and anti-smooth muscle antibodies. Repeated testing for EBV confirmed negative EBV-VCA IgM, positive EBV-VCA IgG (1:1,024), EBV-EA IgG (1:64) and negative anti-EBNA IgG. Liver biopsy showed intralobular hepatitis and mild periportal lymphocyte cell infiltration consisting mainly of CD3+ T cells. Immunohistochemistry was negative for EBV, cytomegalovirus and herpes simplex virus type 1. Autoimmune-type hepatitis was suspected, and treatment with prednisolone (60 mg/day) was started. After transient improvement, liver function tests deteriorated. Since replicating EBV DNA was demonstrated in whole blood, serum, sputum and bone marrow by PCR, high-dose intravenous immunoglobulin, acyclovir and later ganciclovir were added. Liver enzymes further increased (20–100 times upper limit of normal) and EBV PCR remained positive in serum throughout therapy; however, liver biopsies 2 and 4 months after initiating the therapy did not reveal EBV by different methods. One year after onset of liver disease, the boy developed liver failure, interstitial pneumonitis and severe neutropenia and died from candida septicemia. Autopsy was not done.

Materials and Methods

Flow Cytometric Analysis of Peripheral Blood Cells

Flow cytometric analysis was performed as previously described [13]. In brief , 90 μl EDTA blood were incubated at room temperature for 10 min with 10 μl of a combination of three directly conjugated monoclonal antibodies: CD3–, CD45RO-FITC (Coulter, Krefeld, Germany), CD25-, CD57-, γ/δ-TCR-FITC (Becton Dickinson, Heidelberg, Germany), CD4-, CD16-, CD56-, CD69-, HLA-DR-PE (Becton Dickinson), CD14-, CD19-, CD8-Tricolor® (Medac, Hamburg, Germany) in optimal dilutions. Blood was lysed in a Q-Prep lysing apparatus (Coulter) and analyzed by flow cytometry within 24 h (FACScan, PAINT-a-GATE data analysis program, Becton Dickinson). For the analysis of subpopulations, gates were set on lymphocytes, monocytes, or lymphocytes and monocytes, respectively.

Cell Culture Assays

Short-time cell culture was performed with peripheral blood mononuclear cells (PBMC) isolated from heparinized blood by density centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden). The cells were cultured in 96-well culture plates (NUNC, Wiesbaden, Germany) at 37°C in RPMI medium (Gibco, Eggenstein, Germany), supplemented with *L*-glutamine, penicillin/streptomycin and 10% human AB serum. Atypical $CD4^{low}$ cells were enriched by 1 h incubation of PBMCs in culture flasks to remove monocytes by adhesion. The nonadherent cells were cultured in 96-well culture plates.

Results

Flow Cytometry

Flow cytometric analysis of the boy's peripheral blood cells revealed a major subpopulation (20.3%) of atypical cells in the lymphocyte gate. These cells were CD3+; but the level of CD4 expression was very low, comparable to that of monocytes. They were HLA-DR+ and CD45RO+, but CD8–, CD14–, CD19–, CD16–, CD56–, CD57–, CD25– and γ/δ-TCR–. The remaining PBMC consisted of 19.9% monocytes with regular FSC/SSC characteristics, 7.5% 'normal' $CD4^{high}$ T cells, 29% CD8+ T cells, 10% B cells and 9.3% NK cells (fig. 1). The flow cytometric analysis was repeated 4 weeks later with no significant change in any cell population.

Cell Culture

Using whole PBMCs for cell culture, 'clusters' of different cells could be seen for about 20 days, but then disintegrated (fig. 2A). After 24 h in culture, isolated atypical $CD4^{low}$ lymphocytes became adherent, enlarged and acquired multiple granules and dendrites (fig. 2B). When removing the cells from the culture dishes, all cells disintegrated. Analysis in 96-well microtiter plates showed a loss of CD3 and CD4. The cells remained negative for CD8 and CD14, but stained brightly for HLA-DR. No cell line of these atypical $CD4^{low}$ lymphocytes could be maintained.

Discussion

Fatal infectious mononucleosis occurs as an X-linked lymphoproliferative syndrome (XLP) in males [11, 14, 15]. In addition, Sakamoto et al. [16] and others described a sporadic form of fatal mononucleosis in previously healthy males and females. The median age at death of patients with sporadic fatal mononucleosis is about 10 years, in contrast to only 2.5 years in children dying from XLP [10]. Our patient died at the age of 10 years, 1 year after acute infectious mononucleosis from EBV-induced immunodeficiency with severe liver and pulmonary disease. The patient had no siblings and the family history was negative for fatal infections or deaths of unknown cause during childhood. Bone marrow aspiration showed no signs of a hemophagocytic syndrome. Sonography and CT scan did not reveal any development of lymphoma. Serology showed persistently high titers of EBV-VCA-IgG and EBV-EA-IgG. EBV DNA was detected by PCR in whole blood, serum, sputum and bone marrow. EBV-VCA IgM was negative, but a false-negative result at the onset of disease due to the presence of exceedingly high titers of EBV-VCA IgG masking IgM cannot be excluded. Throughout the whole course of disease, anti-EBNA remained negative, thus indicating chronic EBV infection without adequate immune response. Therefore, we suspect that our patient represents one of the sporadic cases of fatal chronic EBV infection. Hepatopathy was the pre-

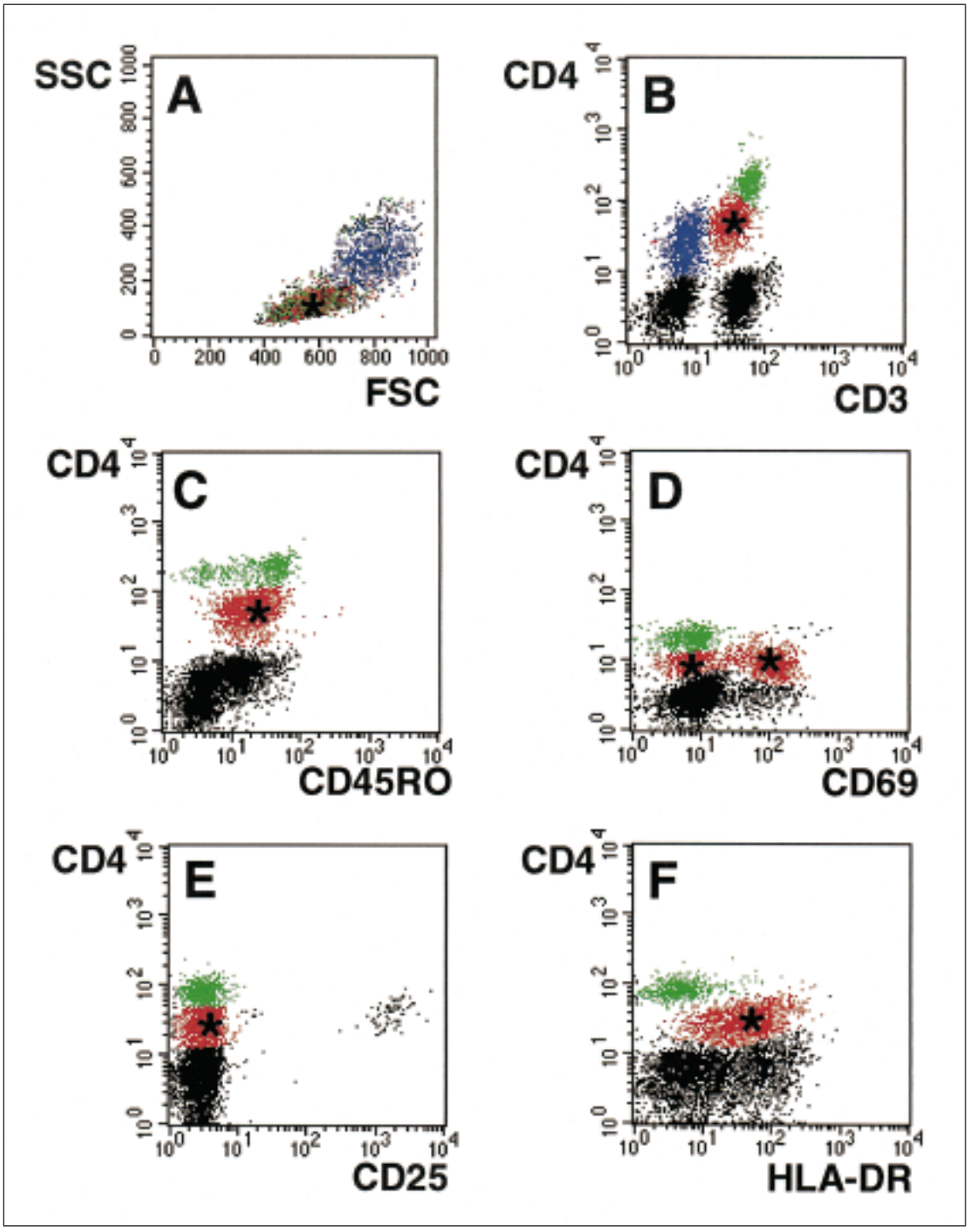

Fig. 1. Flow cytometric analysis of peripheral blood cells. **A,B** Gate set on lymphocytes and monocytes. **A** FSC/SSC characteristics of the PBMCs. **B** Gated mononuclear cells: monocytes (blue), typically low $CD4^{low}$+. Two populations of CD3+ cells: green normal $CD4^{high}$ T lymphocytes, red atypical $CD4^{low}$+ cells. **C–F** Gate on lymphocytes. Expression of CD45RO (**C**), expression of CD69 on most atypical cells (**D**), HLA-DR (**F**) on virtually all atypical cells, but no expression of CD25 on these cells (**E**).

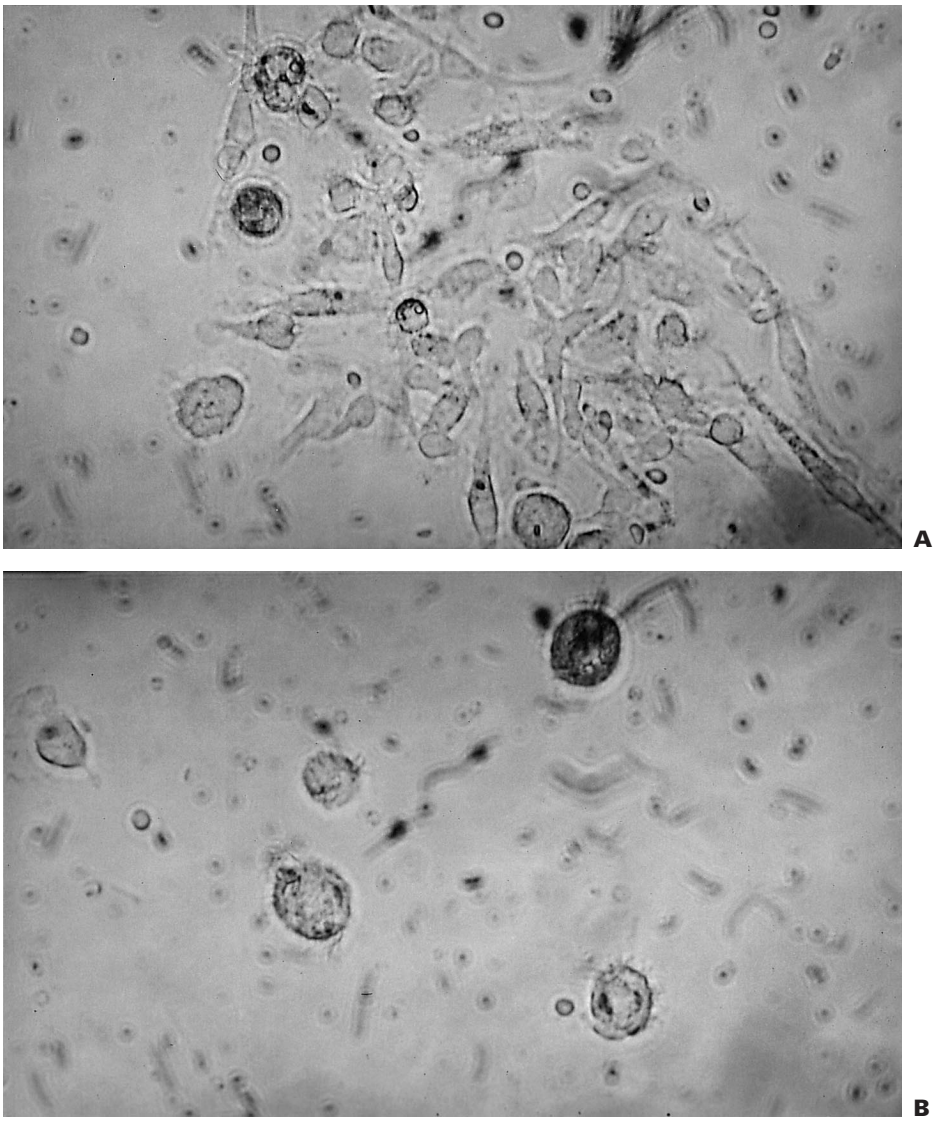

Fig. 2. A Short-time cell culture of isolated PBMC: 'clusters' of different cells which could be seen for about 20 days but then disintegrated. ×220. **B** Enriched atypical $CD4^{low}$ cells: within 48 h, the cells became adherent and developed granules and small dendrites (–>). ×360.

senting manifestation of the boy as has been described in the literature for most similar cases [10]. A direct hepatocytopathic effect of the virus has been suggested [17]. However, the only proven receptor for EBV (CD21 or C3d) is confined to B cells, T cells and dendritic reticulum cells, although for certain anti-CD21 antibodies, a staining on different other cells, including hepatocytes was found [18]. Vento et al. [19] suggested that EBV triggers an autoimmune reaction directed against hepatocytes in susceptible persons. This concept is supported in our patient by the absence of virus DNA in all three liver biopsies, whereas EBV DNA could be seen in sputum, peripheral blood and bone marrow.

There are only few data describing alterations in the lymphocyte subpopulations of peripheral blood of patients with chronic EBV infection. One case report described an EBV-induced CD30+ NK cell-type malignancy. Interestingly, this patient also had a remarkable hypogammaglobulinemia. The atypical cells in our patient were negative for NK-cell markers such as CD16, CD56 and CD57 [20]. Three cases of XLP described by Sullivan and Woda [11] showed largely increased CD8 T cells, increased B cells but diminished CD4 T cells. Further, the amount of CD4 expressed at the cell surface is very restricted in vivo. Thus, even in HIV infection, CD4 expression on T cells in peripheral blood remains high [6]. A rare exception is the description of $CD4^{low}$, CD8+ HLA-DR+ lymphocytes in patients with acute infectious mononucleosis [12]. In vitro, CD4 can be downregulated by strong activation, e.g. with phorbol myristate acetate (PMA) or anti-CD3 antibodies [21, 22].

Therefore the downregulation of CD4 in our patient may be due to a specific activation by EBV or an indirect activation of the immune system. This is supported by the expression of other markers of activation on these cells. Sixty percent of the cells were positive for CD69, a marker which is expressed in the early phase of lymphocyte activation. In addition, all cells were positive for HLA-DR, which is usually expressed in a later phase of activation. Recent work indicates that cells of the T cell lineage can be infected with EBV via a CD21-like molecule [23, 24]. However, altered CD4 expression has not been described in EBV-infected T cells. In peripheral blood, monocytes and dendritic cells also show a low expression of CD4. Some features give evidence that these cells may be related to dendritic cells: low expression of CD4, high positivity for HLA-DR, but no CD14 expression. They fit perfectly into the lymphocyte gate, but adhere to plastic dishes and establish tiny dendrites after long-term in vitro culture. On the other hand, these cells strongly express CD3, which is not found on dendritic cells [26] (fig. 1). However, since most methods to isolate dendritic cells use a T cell depletion step, e.g. with monoclonal anti-CD3 antibodies, CD3+ dendritic cells may be lost for analysis. We cannot exclude the possibility that these CD3+, $CD4^{low}$ cells were induced by therapy with acyclovir or ganciclovir although such effects have not been reported. It seems unlikely that these cells represent true neoplasms because EBV-induced leukemia or lymphoma were excluded by bone marrow aspiration and CT scan. In summary, we suggest, that the CD3+, $CD4^{low}$ cells of the patient represent either strongly activated T cells with downregulated CD4 expression or a hitherto unknown subtype of dentritic cells.

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