Cell-mediated Immune Reactions in Three Patients with Malignant Lymphoproliferative Diseases in Remission and Abnormally High Epstein-Barr Virus Antibody Titers

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Abstract

Two patients with Hodgkin’s disease in remission and one chronic lymphatic leukemia patient with extraordinarily high anti-Epstein-Barr virus (EBV) (viral capsid antigen) antibody titers (>10,000) were selected to study a spectrum of cell-mediated immune responses, including natural killer, interferon-boosted killer, antibody-dependent lymphocytotoxicity, and T-cell-mediated reactions. The purpose was to compare these reactions in patients with immunosuppression and a high EBV load who can hold their EBV-carrying cells under control with the corresponding reactions in patients with EBV-carrying lymphoproliferative disease. In contrast to the latter group, the three patients of the present study showed a less profound and less general suppression of the immune responses. Multiple effector mechanisms probably safeguard against the proliferation of EBV-transformed B-cells. Clinically manifest EBV-carrying lymphoproliferative disease occurs only in very severe immunodeficiencies effecting multiple effectors.

Introduction

In certain immunosuppressive conditions, striking changes in response to EBV can occur (2, 16, 17; for review, see Ref. 12). The most frequently recorded change is an increase in antibody levels to the antigens associated with the viral cycle, VCA and EA, while there is either no increase or a decrease and even a lack of antibodies to EBNA.

Whatever the mechanism leading to these changes, the results strongly suggest that the controls that regulate antibody production against the viral antigens are fundamentally different from those that regulate the production of anti-EBNA. Conceivably, different effectors are responsible for maintaining the level of EBNA-releasing cells (i.e., EBV-transformed cells with a proliferative potential that are not engaged in a viral cycle) and of cells that are in the viral cycle itself, with synthesis and release of EA and VCA. Noteworthy is that NK sensitivity of EBV-carrying cells increases markedly when cells enter the viral cycle (3).

In spite of the elevated antibody titers that suggest a high load of virus and virally infected (transformed) cells, most patients in the categories so far mentioned can still control the proliferation of their EBV-carrying cells, since they are not characterized by any clinically evident lymphoproliferative disease induced by EBV. This situation must be distinguished from a variety of recently identified immunodeficient patients who develop more or less malignant proliferations of EBV-carrying cells. They include fatal infectious mononucleosis, chronic infectious mononucleosis, phenotypes of the X-linked lymphoproliferative syndrome, “immunoblastic sarcomas” in renal transplant recipients, and malignant lymphoproliferative disease in ataxia telangiectasia (7, 36, 39). A detailed analysis of the immune effector mechanisms that keep the proliferation of EBV-transformed B-lymphocytes under control is of great interest, both for the understanding of normal surveillance mechanisms and for the study of the EBV-associated proliferative diseases. Since EBV is harbored by B lymphocytes, themselves a component of the immune system, such studies may elucidate important interactions of networks within the immune system.

As a first step towards such studies, we have analyzed a variety of cell-mediated immune reactions towards EBV-transformed cells and EBV antigens in 3 immunologically compromised patients: 2 with HD and one with CLL. The 2 HD patients were free of clinically detectable tumors and the CLL patient was in partial remission at the time of the study. All 3 patients had extraordinarily high antibody levels against VCA and EA, whereas antibodies to EBNA were not elevated. They also showed defects in the lymphocyte functions.

Materials and Methods

Donors. Fifty-mi volumes of heparinized blood were obtained from patients and EBV-seropositive and -seronegative healthy controls.

Three patients, A. W., L. G., and A. E. (see "Case Reports"), who developed high antibody titers against EBV VCA were selected for the study. Four bleedings were performed over a 5-month period. Antibody titers against VCA, EA, and EBNA were obtained repeatedly before and again at least twice after initiation of the present study.

EBV Antibody Tests. IgG, IgA, and IgM antibodies to EBV VCA and to D or R were titrated by the indirect immunofluorescence techniques as described (8, 13, 32). Antibodies to EBNA were measured by anticomplement immunofluorescence (11, 37).

Spontaneous Mitogen- and Antigen-induced DNA Synthesis. The method was described in detail previously (15). Lymphocytes were purified from defibrinated venous blood by sedimentation in gelatin. After ingestion of carbonyl iron, phagocytic cells were removed with a magnet. For enumeration of T-lymphocytes (18), residual RBCs were removed by sedimentation through a Ficoll-isopaque gradient. Lymphocytes (10^6/ml) were suspended in RPMI 1640 with 15% heat-inactivated pooled human AB serum. Spontaneous lymphocyte DNA synthesis was measured by the incorporation of [3H]thymidine as described during the first day of culture. Mitogen (pokeweed mitogen and concanavalin A) or antigen (PPD) stimulation was measured on the third day of culture.

Skin Tests. These tests were performed as described before (15). Briefly, 0.1 ml PPD of tuberculin (Statens Seruminstitut, Copenhagen, Denmark) was injected intradermally on the volar surface of the right forearm. Skin reaction was evaluated 48 hr later by measuring the crossed diameters of induration and erythema. An induration of 6-mm diameter or more was considered positive. Collection of lymphocytes for in vitro studies always preceded skin testing.

LMI. The direct agarose microdroplet assay was carried out according to McCoy et al. (28), with minor modifications. Twenty x 10^6 washed buffy coat leukocytes were mixed with 135 µl nutrient agarose medium, containing an equal volume of 2× RPMI 1640 supplemented with 20% FCS (Grand Island Biological Co., Springfield, Va.) and 0.4% agarose. Two-µl droplets of the suspension were placed into migration chambers (Sterilin, Teddington, England). After solidification of the droplets, the chambers were filled with medium (control) or with medium supplemented either with 0.5 µg purified PHA (Wellcome, Hartford, England) per ml or with the optimal concentrations of the appropriate EBV antigen-containing extracts, as described previously (41). The chambers were covered and placed in a humidified 5% CO_2 atmosphere at 37°C. After 18 to 24 hr, the migration areas were projected and measured as described by Weese et al. (43) and a migration index was calculated as follows:

\[
\text{Migration index} = \frac{\text{mean of triplicates in the presence of antigen}}{\text{mean of control triplicates}}
\]

A migration index value of less than 1.0 indicates LMI.

Cell extracts were prepared from the cell lines listed in Table 1. Cells were grown in RPMI 1640 with 10% heat-inactivated FCS and antibiotics and harvested weekly for antigen preparation. Partially purified EBNA was prepared and tested as described previously (24, 41).

The induction of P3HR-1 cells for the production of VCA and EA was carried out with n-butyrate (3 mM concentration; 72 hr) as described (19). Induction resulted in 27 to 46% EA- and 10 to 30% VCA-positive cells.

Viral Transformation and Outgrowth Inhibition Test. Cell supernatants from the virus producer line B95-8 (29) were used as a source of transforming virus. Supernatants used contained 2 x 10^6 infectious units, as measured by EBNA induction in Ramos cells (21). Non-T-lymphocytes (10^6) were incubated with 1 ml undiluted virus preparation for 1 hr at 37°C. Excess virus was removed, and the cells were washed once with tissue culture medium after the incubation period. Outgrowth inhibition was assayed according to a modification of the methods described by Thorley-Lawson et al. (42) and Moss et al. (30). The infected cells were reconstituted with T-cells and incubated in bottles or microplates as described below. Some infected B-cells were incubated without T-cells in small Falcon tubes (5 ml; Falcon Plastics, Los Angeles, Calif.) to allow development of cell lines to be used in autologous cytotoxicity experiments.

For the outgrowth inhibition experiments, heparinized blood was separated on Ficoll-isopaque and on nylon wool. The cells recovered after passage through nylon were highly enriched in T-cells. The B-cell-enriched nylon-adsorbed T-cells were recovered by incubating the columns in 100% FCS for 30 min as described previously (44). Following infection with B95-8 virus, B-cells were reconstituted with the T-cells at a 9:1 T-cell:B-cell ratio. The reconstituted mixture (0.2 ml), at concentrations of 2.5 x 10^5/ml, 0.5 x 10^5/ml, and 0.2 x 10^5/ml, was placed in flat-bottomed micro wells (10 wells/dilution). Growth was evaluated every 3 to 7 days by the following score: ++, single blasts; +++, groups of 3 to 4 blasts; ++++, large clumps with blasts growing out of the clumps; +++++, overgrowth; --, no growth or blasts.

An average result of one + will be represented in the figures as 25% outgrowth (75% inhibition), an average of 4 + + will be represented as 100% outgrowth (0% inhibition). As controls, we used B-cells infected alone (100% outgrowth after 14 days of culture) and uninfected B-cells mixed with T-cells (0% outgrowth after 14 days of culture).

Parallel bottles were prepared containing infected cells plus T-cells (1:9 ratio), 10^6 cells/ml in 10 ml. These bottles were fed every 4 to 7 days. Living-dead cells, surface immunoglobulin-positive cells, and sheep erythrocyte-rosetting cells were counted at regular intervals. On the 14th day of culture, T-lymphocytes were separated by sheep erythrocyte rosetting and Ficoll-isopaque sedimentation and used for cytotoxicity tests.

Cytotoxicity Tests. Fresh lymphocytes (after Ficoll-isopaque sedimentation and macrophage depletion) and T-lymphocytes obtained after 14 days of culture with autologous EBV-infected B-cells were used as effectors. Cytotoxic activity was tested in a 6-hr 51Cr release assay as described previously (1). The targets included K562 cells, an EBV-negative erythroid leukemia line that is known to be highly sensitive to NK cell-mediated killing, Daudi cells, an EBV-carrying Burkitt lymphoma line, and autologous and allogeneic LCLs established by EBV infection of normal lymphocytes in vitro.

Table 1: EBV antigen content of cell lines used for extracts or EBNA preparation for LMI studies

<table>
<thead>
<tr>
<th>Designation</th>
<th>Origin</th>
<th>EBV genome</th>
<th>EBNA</th>
<th>EA</th>
<th>VCA</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BJAB</td>
<td>Burkitt-like lymphoma</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>22</td>
</tr>
<tr>
<td>BJAB/B95-8</td>
<td>EBV-converted subline of BJAB (B95-8 subtrain)</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>6</td>
</tr>
<tr>
<td>Ramos</td>
<td>American Burkitt lymphoma</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>21</td>
</tr>
<tr>
<td>P3HR-1</td>
<td>African Burkitt lymphoma</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>14</td>
</tr>
<tr>
<td>Raji</td>
<td>African Burkitt lymphoma</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>5</td>
</tr>
</tbody>
</table>

IFN Treatment of Effector Lymphocytes. Parallel lymphocyte samples were incubated for 3 hr in 1 ml RPMI 1640 with 10% FCS. One thousand units of IFN were added to one aliquot. The IFN (kindly provided by Dr. G. Bodo) was prepared from Namalwa cells, induced by Sendai virus. The preparation contained 2.2 x 10^4 units/mg protein. It had been purified by Bodo as described previously (4).

Marker Studies. To detect T-cells, sheep erythrocyte rosetting was performed according to Jondal et al. (18). For isolation of erythrocyte-rosetting cells, the suspension was layered on Ficoll-isopaque and spun at 500 x g for 20 min. The pellet was collected, and the...
erthyrocytes were lysed by 6 sec of treatment with distilled water. Surface immunoglobulin-positive cells were detected by direct immunofluorescence with rabbit anti-human polyclonal immunoglobulin (Dako, Copenhagen, Denmark).

ADCC: For the ADCC assay, Raji cells were grown in RPMI 1640 plus 10% heat-inactivated (56° for 30 min) FCS. EBV from P3HR-1 cultures, provided by the Division of Cancer Cause and Prevention, National Cancer Institute, was kept at -70° until used. To induce the ADCC-sensitive membrane antigen, 5 × 10⁶ Raji cells were incubated with 1.0 ml of an appropriate virus dilution at 37° for 1 hr. The cells were then resuspended in RPMI 1640 plus 10% FCS to a concentration of 1 × 10⁷ cells/ml. The infected cultures were then incubated at 37° for 24 hr. Cultures harvested at this time usually contained 30 to 50% membrane antigen-positive cells as reported previously (33).

To prepare effector cells, blood from individual baboons was collected by venipuncture into a syringe containing heparin. The blood was diluted 1:3 in growth medium, and then the leukocyte fraction was separated on Ficoll-Hypaque gradients (LSM solution; Litton Bionetics, Inc., Kensington, Md.). These lymphocytes were used routinely as effector cells in the ADCC assay. Sera were assayed for ADCC activity against 51Cr-labeled EBV-infected or uninfected Raji cells by using a microassay as described (34). The ratio of lymphocytes to target cells was 100 to 1. The microplates were incubated at 37° for 3 to 4 hr. Cytotoxicity for the lymphocyte-serum mixtures was calculated as reported previously (35). For calculating the ADCC, the cytotoxicity figure for lymphocytes incubated in the presence of an antibody-negative control serum was subtracted from that induced by the corresponding dilution of the test serum. The statistical significance was determined by Student's t test. The final serum dilution showing an increase in lymphocyte cytotoxicity significant at p < 0.05 was chosen as the serum titer.

Case Reports

Patient A. W. This young girl presented in September 1966 at the age of 14 with a large progressive swelling in the left supraclavicular area. Chest roentgenogram showed moderate widening of the upper mediastinum. The diagnosis of HD was established by aspiration biopsy. Local field irradiation to the left neck and mediastinum led to the disappearance of the lymphadenomegaly.

The patient remained symptom-free until June 1970 when a right supraclavicular recurrence appeared. Local 60Co-irradiation to the neck and the axillas resulted in prompt regression. She was in remission until May 1975 when chest X-ray showed a lung lesion close to the right hilar region. Local irradiation caused regression of the lung lesion. Diagnostic laparotomy with splenectomy was performed, and no disease was observed in the spleen or in biopsy specimens from the liver and abdominal lymph nodes. Despite the negative findings, inverted Y-field irradiation was given.

A second pulmonary recurrence was found on routine examination in March 1976. She was started on combined chemotherapy with nitrogen mustard-vincristine-procarbazine-prednisone and was later changed to 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea-vincristine-procarbazine-prednisone. Chemotherapy was continued with a total of 10 courses and finished in November 1977.

In February 1978, there was progression in the lung, and the patient was given local irradiation, combined with bleomycin. She complained of fatigue at the completion of the treatment. Two months later, her previously normal liver function tests became abnormal. Two weeks later, she developed fever very abruptly. On admission to the hospital, *Streptococcus pneumoniae* was cultured from her blood. Treatment with benzyl penicillin resulted in prompt improvement. Computerized tomography including the liver, liver scan, and chest X-ray were normal. Needle biopsies from the liver showed no significant abnormality. The liver function gradually became normal in April 1979.

When the EBV study was performed, the patient was in complete remission and working. Electrolyphoresis, liver function tests, and chest X-ray were normal.

Patient L. G. This 68-year-old woman was hospitalized in June 1976 for glomerulonephritis. There was a history of weight loss but not of fever or night sweats. Routine clinical examination revealed cervical, axillary, and inguinal lymphadenomegaly, which the patient had noticed for 1 year. Chest X-ray was normal. Radiogram of the abdomen showed a slightly enlarged spleen. The bone marrow was massively infiltrated with small mature lymphocytes as in CLL. Biopsy of a cervical node, on the other hand, showed diffuse infiltration of poorly differentiated lymphocytes of a type often seen in lymphocytic lymphoma. Hemoglobin was 93 g/liter with 20% reticulocytes; WBC was 22.0 × 10⁹/liter with 90.5% small mature lymphocytes. A monoclonal k light chain was found in the urine. Liver function tests were normal. The disorder was classified as CLL because of the bone marrow picture and the presence of leukemia cells in the blood, although the lymph node picture was atypical.

Treatment was started in August 1976 with intermittent courses of Cyclophosphamide-Oncovin-Prednisone and later changed to Prednimustine. The WBC fell dramatically to 1.1 × 10⁹/liter. Meanwhile, the lymph nodes gradually shrunk. Treatment was discontinued in November 1976. Bone marrow showed improvement but not complete remission.

In March 1978, the lymphocyte count and the peripheral lymph nodes were found to be increasing. Prednimustine treatment was reinstituted in December 1978. The patient responded again with normalization of the peripheral blood picture and shrinkage of the enlarged nodes. The treatment was stopped in July 1979, and the patient has not required any further treatment.

At the time of the EBV study, the patient was in partial remission. Mature lymphocytes dominated the bone marrow but not to the same degree as on admission. The peripheral blood picture was normal, and the lymph nodes were only slightly enlarged.

Patient A. E. A 42-year-old man admitted in March 1975 with weight loss for 6 months and fever for 1 month. A large mass was found in the right groin. Biopsy showed HD of mixed cellularity. Chest X-ray was normal. Lymphangiography revealed large paravertebral lymph nodes with abnormal structures. The liver and spleen were slightly enlarged. He was classified clinically as Stage II B.

The patient was treated with inverted Y-fields (42 Gy) from April to June 1975. The treatment resulted in improvement of his general condition.

Diagnostic splenectomy was performed in September 1975, and the spleen was found to be involved with HD whereas a liver biopsy was normal. Prophylactic mantle treatment (40 Gy) was given in October 1975.

The patient has remained free of tumor. At the time of EBV study, chest X-ray, computerized tomography, liver scan, bone marrow, and peripheral blood cell counts were normal. The patient was working full time and felt well.

Results

**EBV-specific Serology**

Chart 1 shows the EBV-specific antibody spectrum and titers of the 3 patients.

The first serum from A. W. (Chart 1A) was collected 3 years after the first diagnosis of HD when she still had no antibodies to EBV. She was still EBV antibody negative 4 years later. The next serum obtained in February 1978, when the patient was 26 years old, revealed seroconversion, possibly in the recent past because the anti-EBNA titer was low (1:10). However, IgM antibodies to VCA were not detected. The titers of VCA- and D-
specific IgG and IgA antibodies, as well as anti-EBNA, rose 4- to 8-fold during the next 3 months. During this period and concomitantly with the antibody rise, the patient had an episode of hepatitis. Subsequently, a streptococcal septicemia developed. During the subsequent maintained remission, all antibody titers remained essentially constant, except for D-specific IgA which slowly disappeared.

The first available serum from L. G. (Chart 1B) was obtained at the time of diagnosis of CLL. It showed unusually high titers of IgG and IgA antibodies to VGA and D, but the anti-EBNA level was within the normal range. This serum and 2 subsequent sera reacted also in the test for IgM antibodies to VGA at a titer of 1:40 (not shown), but this reaction was due to rheumatoid factor and removed by adsorption with IgG-coated latex particles (10). After cessation of therapy, some of the antibody titers rose 2- or 4-fold, possibly with progression of the leukemia, but they declined again following therapy.

The first sera from A. E. (Chart 1C) were obtained at the time of the diagnosis of HD and towards the end of total nodal radiation therapy. The IgG antibodies to VGA rose from 1:80 to 1:320 within one month and before any treatment was started, and anti-R became detectable at a titer of 1:10. No IgA antibodies to VGA were found at this early stage, but they were present at substantial titers in the next serum collected 23 months later when the IgG antibodies to VGA, D, and R had risen to 1:5120, 1:80, and 1:320, respectively. Also, this patient developed low levels of rheumatoid factor which registered nonspecifically in the test for VCA-specific IgM antibodies. Although the patient remained free of clinical tumor more than 4 years, the antibody spectrum and titers changed very little. Because the patient had considerable titers of antibodies to both the D and R components, it was difficult to clearly differentiate between the 2 on some occasions.

ADCC

Antibody as measured by the ADCC assay varied among the 3 patients, but on repeated tests from the same patient, it was stable. The ADCC titers in the 3 serum samples collected from A. E. at 1-month intervals were all 15,360 which is considered high in this test. The titers in the 2 serum samples from L. G., also collected at 1-month intervals, were 15,360, while the ADCC titer on one serum sample from A. W. was 720 which is considered low for this test.

Tests for General Lymphocyte Reactivity

Spontaneous and Mitogen-Induced Lymphocyte DNA Synthesis and T-Lymphocyte Levels in Patients and Controls

<table>
<thead>
<tr>
<th>Patient</th>
<th>Date</th>
<th>Spontaneous DNA synthesis (log cpmp)</th>
<th>PWM</th>
<th>Con A</th>
<th>PPD (2.5 μg/ml)</th>
<th>log cpmp (%)</th>
<th>T-Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. W.</td>
<td>10/6/79</td>
<td>1.889 1.811 2.737 2.893</td>
<td>3.029 3.125 1.930 3.27</td>
<td>67</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>L. G.</td>
<td>12/14/79</td>
<td>2.158 2.073 2.674 2.860</td>
<td>2.864 2.941 1.905 2.97</td>
<td>53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. E.</td>
<td>1/2/80</td>
<td>1.603 1.391 2.642 2.564</td>
<td>2.667 2.747 2.456 2.84</td>
<td>53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. E.</td>
<td>1/16/80</td>
<td>1.809 1.548 1.751 1.874</td>
<td>2.041 2.052 1.529 3.30</td>
<td>65</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 57)</td>
<td>1.619 1.916 2.133 2.071</td>
<td>2.173 2.305 1.328 3.39</td>
<td>70</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2

*PWM, pokeweed mitogen; Con A, concanavalin A.*

*57 Fifty-seven healthy donors (mean age, 41 years; range, 20 to 91 years).*

*± Mean ± S.E.*

Spontaneous Mitogen- and Antigen-Induced Lymphocyte DNA Synthesis. The spontaneous lymphocyte DNA synthesis was increased in Patient A. W. but essentially normal in the other 2 patients (Table 2). A more severe impairment of mitogen (pokeweed mitogen and concanavalin A)-induced lymphocyte activation was found in Patient A. E., while Patients A. W. and L. G. displayed moderate defects. The *in vitro* response to PPD stimulation was normal in L. G. and impaired in A. W. and A. E.
Marker Studies and PPD Skin Test. Patients A. W. and Å. E. showed no delayed skin hypersensitivity to PPD. Patient L. G. who had a normal in vitro response to PPD also showed a positive response in vivo. Relative and total T-lymphocyte counts were essentially within the normal range in all 3 patients.

Spontaneous Cytotoxic Activity

Spontaneous cytotoxic activity of blood lymphocytes was measured against the highly NK-sensitive K562 target cell line. Four tests were performed on each patient at monthly intervals (Chart 2). The normal range is indicated by the horizontal line (mean percentage of 51Cr release obtained in 20 tests performed with blood lymphocytes from 10 normal controls over the same time period) and by the parallel dotted lines (S.D.). The cytotoxic activity against K562 was largely within the normal range in all 3 patients. The low cytotoxicity obtained in the first test with L. G. cells was probably due to a dilution of the effectors by the leukemic cells present in peripheral blood (63% of the lymphocytes had surface immunoglobulin). In the following tests, nylon-passed cells were used as effectors. With the exception of one test with Å. E. cells, there was no significant variation in cytotoxic activity in any of the patients during the period of the study.

LMI Tests

Previously, we (39) have described an EBV-specific LMI system, responsive to the extracts of EBV-containing cells and to EBNA, respectively. This method has been used in the present study, with particular attention to the possibility of distinguishing between the sensitization state of the patient to the EBV antigens associated with the viral cycle (EA and/or VCA), on the one hand, and to the transformation-proliferation-associated EBNA, on the other hand. As previously, LMI by PHA was used as a positive control for a general assessment of lymphocyte reactivity.

Table 1 summarizes the EBV antigen content of the cell extracts. In addition, EBNA partially purified by the method of Luka et al. (24) was also tested. Table 4 summarizes the LMI indices for healthy seronegative and seropositive donors and for the 3 patients. Healthy seronegative donors showed no differences in LMI between EBV-negative and -positive cell lines or between EBNA and mock EBNA. The healthy seropositive donors showed significant LMI following exposure of their leukocytes to the extracts of the EBV genome-carrying cell lines and to partially purified EBNA but not to mock EBNA.

The 3 patients reacted as follows: A. W. reacted normally to PHA in one of 2 tests and weakly in the other. Buffy coat leukocytes of Å. E. responded well to 1 μg PHA per ml in 2 tests. The same was found with the T-cell-enriched buffy coat cells of L. G. (Table 4, Column A). This indicates that the lymphocytes of all 3 patients could produce LMI factor in the response to PHA, although to different degrees.

None of the leukocytes responded to cell extracts from the EBV-negative BJAB (Column B). Treatment of BJAB with n-butyrate, a powerful inducer of EBV antigens in EBV-carrying cell lines, did not change the inability of this line to induce a LMI reaction.

Extracts of the EBV genome-carrying nonproducer lines BJAB/B95-6 and the EBV producer P3HR-1 line inhibited the migration of leukocytes from EBV-seropositive but not -seronegative individuals (Columns C and D). It should be noted that BJAB/B95-8 is an in vitro EBV-converted subline of the originally negative BJAB and Ramos line (Table 1). The difference between the EBV-negative lymphoma lines and their own EBV-converted sublines is exclusively referable to the presence of the viral genome. These cells express EBNA (37) but none of the other EBV-specific antigens. Correspondingly, partially pur-
### Table 4

**LMI tests**

<table>
<thead>
<tr>
<th>Patients</th>
<th>Dates of experiments</th>
<th>PHA (1 µg/ml)</th>
<th>EBV-negative cell line BJAB</th>
<th>BJAB/B95-8 (EBNA+)</th>
<th>P3HR-1 (VCA+, EA+, EBNA+)</th>
<th>P3HR-1 + n-butyrate (VCA+++, EA++++, EBNA+)</th>
<th>Mock EBNA&lt;sup&gt;c&lt;/sup&gt; (10 µg/ml)</th>
<th>EBNA&lt;sup&gt;d&lt;/sup&gt; (10 µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy seronegative donors</td>
<td>12/14/79</td>
<td>0.47</td>
<td>0.85</td>
<td>0.95</td>
<td>0.83</td>
<td>0.70</td>
<td>0.92</td>
<td>0.83</td>
</tr>
<tr>
<td></td>
<td>1/16/80</td>
<td>0.78</td>
<td>1.17</td>
<td>1.05</td>
<td>1.10</td>
<td>0.76</td>
<td>1.17</td>
<td>0.92</td>
</tr>
<tr>
<td>Healthy seropositive donors</td>
<td>11/28/79</td>
<td>0.56</td>
<td>0.89</td>
<td>1.0</td>
<td>0.85</td>
<td>No migration</td>
<td>0.82</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>1/30/80</td>
<td>3/28/80&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.91</td>
<td>1.21</td>
<td>ND&lt;sup&gt;d&lt;/sup&gt;</td>
<td>1.38</td>
<td>1.32</td>
<td>1.29</td>
</tr>
<tr>
<td></td>
<td>3/26/80&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0.36</td>
<td>0.92</td>
<td>ND</td>
<td>0.60</td>
<td>0.56</td>
<td>0.73</td>
<td>0.46</td>
</tr>
<tr>
<td>A. W.</td>
<td>11/14/79</td>
<td>0.45</td>
<td>0.80</td>
<td>ND</td>
<td>0.40</td>
<td>0.56</td>
<td>0.80</td>
<td>0.52</td>
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<td></td>
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<td>0.39</td>
<td>0.88</td>
<td>0.75</td>
<td>0.57</td>
<td>0.64</td>
<td>0.98</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>1/16/80</td>
<td>ND</td>
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<td>0.61</td>
<td>0.66</td>
<td>0.61</td>
<td>1.15</td>
<td>0.59</td>
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</tbody>
</table>

**Notes:**
- The migration index = mean of triplicates with antigen/mean of control triplicates.
- The cell lines, listed in Table 1, were used at a concentration of 50 µg protein per ml.
- Prepared from Ramos.
- Prepared from Raji.
- Mean ± S.E.
- Numbers in parentheses, number of donors.
- The ratio of the granulocytes in the buffy coat was only 5%, insufficient to detect any migration.
- With buffy coat cells, containing 19% granulocytes.
- After reconstitution of the original buffy coat (see Footnote h) with autologous T-cell-enriched (nylon wool-passed) population in 2:1 ratio.
- ND, not done.

<sup>a</sup> The migration index = mean of triplicates with antigen/mean of control triplicates.

<sup>b</sup> The cell lines, listed in Table 1, were used at a concentration of 50 µg protein per ml.

<sup>c</sup> Prepared from Ramos.

<sup>d</sup> Prepared from Raji.

<sup>e</sup> Mean ± S.E.

<sup>f</sup> Numbers in parentheses, number of donors.

<sup>g</sup> The ratio of the granulocytes in the buffy coat was only 5%, insufficient to detect any migration.

<sup>h</sup> With buffy coat cells, containing 19% granulocytes.

<sup>i</sup> After reconstitution of the original buffy coat (see Footnote h) with autologous T-cell-enriched (nylon wool-passed) population in 2:1 ratio.

<sup>j</sup> ND, not done.
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ified EBNA (Column G) induced significant LMI where lymphocytes from healthy seropositive but not seronegative donors were tested whereas mock EBNA was inert (Column F) (40). 

n-Butyrate is known to induce the synthesis of EA and VCA in a considerable proportion of P3HR-1 cells (23). Nevertheless, extracts of butyrate-induced P3HR-1 cells had no greater LMI effect than those of noninduced P3HR-1 cells or of the nonproducer cells on the migration of leukocytes from healthy seropositives (Column E). Since the leukocytes of these donors show a good response to EBNA and since EBNA is present in all these cell lines, this is not surprising, however.

Of the 3 patients, A. W. showed no response to the extracts of EBV-carrying lines (except butyrate-induced P3HR-1) or EBNA. Thus, with the exception of the significant response to the induced cells, the LMI pattern of this patient corresponded to that of seronegatives, in spite of her extraordinarily high EBV antibody titers (Chart 1A). This was already the case in the first experiment, where the lymphocytes responded normally in PHA. Therefore, the findings indicated a specific defect in the lymphocyte-mediated EBV antigen response of this patient. The fact that the patient did respond to the EA-VCA-enriched butyrate-induced cell extracts in both tests suggested that her response was mainly or exclusively defective in relation to EBNA. This would also fit with her antibody titers, since the latter showed an extraordinary elevation of the response to VCA and a high response to EA, D in particular, whereas the response to EBNA was more within the normal range.

The LMI test with cells from the CLL patient, L. G., was complicated by a low percentage of the migrating granulocytes in the peripheral blood and the diluting effect of the B-cell leukemia cells in the first and second test. In the third test, a reconstituted buffy coat was used with a nylon-passed T-cell-enriched population to obtain a larger fraction of responding cells. This procedure did not influence the reactivity in normal seropositive donors (data not shown). With the reconstituted buffy coat, L. G. responded to the EBV genome-carrying extracts and to the EBNA preparation essentially like a normal donor. The third patient, A. E., showed a normal response to the extracts of all EBV-carrying lines and to EBNA.

### Outgrowth Inhibition

Moss et al. (30, 31) have shown that T-lymphocytes of EBV-seropositive but not -seronegative individuals can inhibit the outgrowth of EBV-transformed autologous LCLs of B-cell derivation. Aspects of this assay are discussed by Rickinson et al. (38) elsewhere in this issue. In a further attempt to define the possible effects of EBV-specific T-cell functions in the 3 patients, we used an outgrowth inhibition test, modified after the method of Moss et al. Briefly, the ability of the patients' T-lymphocytes to inhibit the outgrowth of their own autologous EBV-transformed LCLs was measured in parallel with corresponding tests on normal EBV-seropositive and -seronegative controls. The percentage of inhibition as compared to controls obtained in 2 to 3 experiments performed with each patient are shown in Chart 3. The EBV-seropositive controls showed more than 50% inhibition after 14 days of culture. EBV-seronegative donors gave no only weak inhibition in 9 of 12 tests and a clear inhibition in 3 of 12 tests. Whether this is real or reflects some technical artifact must be left open at this time. The fact remains that the average outgrowth inhibition was 58% in seropositive and 12% in the seronegative control group.

Two of the patients, L. G., and A. E., gave a strong inhibitory response, comparable to normal seropositives. A. W. responded well in one test but poorly in another. On this test occasion, the PHA response was also negative in the LMI test (Table 4, January).

### Cytotoxic Activity against Autologous LCLs

The outgrowth inhibition test, while informative, is cumbersome and subject to variations of the complex tissue culture system. For this reason, we have also measured the cytotoxic activity of various effectors against autologous and allogeneic LCL targets. The effectors included freshly separated lymphocytes with and without IFN stimulation (IAK and NK, respectively) and T-lymphocytes obtained after 14 days of cocultivation with autologous EBV-infected B-cells.

Neither the EBV-seropositive or -seronegative controls nor the patients showed any significant spontaneous killing or IAK against autologous LCLs (Table 5). This is in keeping with our previous experiments (20, 25, 26), showing that freshly established EBV-transformed cell lines are highly resistant to both NK and IAK.

When the lymphocytes were cultured in bottles with autologous EBV-infected B-cells for 14 days, the T-cells of seropositive donors survived better than the T-cells of the seronegatives. Moreover, they often showed a proliferative response (Chart 4, A, B, and C). After 14 days, only few live T-cells were left in the bottles derived from the seronegative donors. In contrast, there was a consistent outgrowth of EBV-positive B-blasts. These data are in agreement with the outgrowth inhibition test described in the previous section.

L. G.'s cells behaved like the cells of EBV-seropositive normal donors. Her T-cells survived well, as a rule. In one test, no live B-cells were present after 12 days in culture (Chart 4E). The T-cells of A. W. and A. E. showed a much poorer survival. Nevertheless, the proliferation of their EBV-transformed B-cells appeared to be under control, since there was no outgrowth of EBV-transformed cell lines (Chart 4, D and F). The T-cells harvested from the bottles were tested for their
cytotoxic activity against K562 and autologous and allogeneic LCLs, passed in vitro for less than 6 months. Chart 5 shows the cytotoxic activity of T-cells derived from the 3 patients, each compared with one EBV-seropositive and one EBV-seronegative control, tested in parallel. In spite of the considerable variability in repeated tests with the cells of the same donor, the EBV-seropositive donors had the highest cytotoxic activity in each experiment, in accordance with the data on T-cell proliferation. Some cytotoxicity was also generated against autologous and allogeneic LCLs in the culture derived from the EBV-seronegative donors and from Patients A. E. and L. G. In contrast, lymphocytes of Patient A. W. showed no or only very low cytotoxicity after cocultivation. This is in line with the insensitivity of the lymphocytes from this patient to IFN boosting, described in an earlier section.

Table 5

<table>
<thead>
<tr>
<th>EBV seropositive</th>
<th>NK</th>
<th>IAK</th>
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<tbody>
<tr>
<td>E. S.</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>Y. T.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>D. J.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>P. R.</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>EBV seronegative</th>
<th>NK</th>
<th>IAK</th>
</tr>
</thead>
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<tr>
<td>I. E.</td>
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<td>0</td>
</tr>
<tr>
<td>L. S.</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>K. T.</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Patients</th>
<th>NK</th>
<th>IAK</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. W.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>L. G.</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>A. E.</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Discussion

Table 6 summarizes the cell-mediated immunity responses of the 3 patients. A. W. showed the most consistent cellular impairment, including the IFN-boosted NK test, LMI with nonproducer and EBNA extracts, and T-cell proliferation and outgrowth inhibition tests in coculture. Weak or no cytotoxicity was generated, in the coculture, against autologous and allogeneic LCLs. The patient had also the lowest ADCC antibody titers.

The other 2 patients showed essentially normal responsiveness in most tests. However, lymphocyte DNA synthesis after stimulation by mitogens was moderately impaired as was the IFN-boosted NK test and T-cell proliferation in coculture from A. E. and low generation of cytotoxicity after cocultivation with EBV-infected B-cells for L. G. and A. E. A. W., who had failed to respond to the LMI effect of EBV-carrying nonproducer extracts and of EBNA and thus behaved like a seronegative rather than a seropositive individual in this test, showed a good response to the butyrate-induced P3HR-1 cell extract, enriched in EA and VCA antigens, associated with the viral cycle. This corroborates the finding that immunosuppressed patients often show depressed or deficient antibody production against EBNA, in parallel with high antibody levels to EA and VCA (12). It supports the notion that cell-mediated immune responses must function normally, in order to provide the immune system with appropriate stimulus for sensitization against EBNA (9). The same critical requirement apparently does not apply for sensitization and antibody production against EA and VCA. This may be related to the fact that EA and VCA producer cells lyse and release their antigen, whereas EBNA-carrying cells are latently infected viable cells with a proliferative potential. Their normal control may require
the full cooperation of the various relevant compartments within the immune system, including both NK and T-cells. Interestingly, the ADCC antibody response was also low in this patient and moderate or high in the 2 other patients with relatively normal cell-mediated immune responses. This suggests that the ADCC antibody response may also be T-cell dependent. In the LMI essay, the same unresponsiveness to the extracts of EBV-carrying nonproducer cells and to EBNA was found in patients in the acute phase of infectious mononucleosis and 2 cases of chronic infectious mononucleosis.

In all these cases, the EA-VCA-enriched extracts inhibited leukocyte migration. The only difference between the mononucleosis patients and A. W. was the fact that none of the mononucleosis patients tested had any detectable anti-EBNA antibodies, whereas A. W. was anti-EBNA positive.

All 3 patients were chosen on the basis of their extraordinarily high anti-VCA titers, from among a material of more than 1000 lymphoma and CLL patients in long-maintained remission. At certain times, they all reached the excessively high anti-VCA (IgG) titer of 5,120 to 20,480. Less than 2% of the patients ever developed antibody titers of this magnitude. As discussed elsewhere in detail (12), EBV titers of this magnitude are indicators of a pronounced immunosuppression. Immunosuppression was also reflected by the cell-mediated immunity test, although most mechanisms tested were only moderately suppressed even in the most clearly affected patients. Apparently, sufficient function is preserved to prevent the proliferation of the EBV-carrying cells.

Elsewhere in this issue, we present evidence (27) to show that patients with chronic infectious mononucleosis and with the X-linked lymphoproliferative syndrome who often fail to control the proliferation of their EBV-carrying cells have severe impairment of both their NK and their T-cell function. The patients with the X-linked lymphoproliferative syndrome in particular also have severe defects in their EBV-related antibody production.

Taken together, the evidence suggests that the impact of the ubiquitous transforming and potentially oncogenic virus, EBV, has selected the human host, in the course of a long-standing symbiosis, for multiple effector control against the proliferation of the transformed cells. Only in a severe immunodeficiency that depresses multiple components of the system does the proliferation progress uncontrolled. Clearly, it is important to define those compartments in this multicomponent system that bear the major responsibility, qualitatively or quantitatively, for what is normally a watertight control mechanism.

Acknowledgments

We give thanks to Sheila Kelly, Marie Adams, Margret Wahlström, Linda van der Waal, and Kent Andersson for skillful technical assistance.

References

13. Henle, W., Henle, G., and Horwitz, C. A. Epstein-Barr virus-specific diag-

Table 6

Schematic summary of the 3 patients' responses

<table>
<thead>
<tr>
<th>Reaction</th>
<th>A. W.</th>
<th>L. G.</th>
<th>A. E.</th>
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<tbody>
<tr>
<td>ADCC against K562</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>NK against K562</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>IAK against Daudi LMI</td>
<td>Weak to normal</td>
<td>Nonresponder</td>
<td>Susceptible</td>
</tr>
<tr>
<td>EBV-carrying nonproducer</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>EBV-carrying producer</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>EBV-producer (butyrate-P3HR-1)</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Outgrowth inhibition</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>T-Cell proliferation</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>B-Cell inhibition</td>
<td>Normal</td>
<td>Normal</td>
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<tr>
<td>Cytotoxicity generated</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
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</table>

a Normal responsiveness refers to the responses of normal EBV-seropositive individuals.
b Coculture with EBV-infected B-cells.
c In coculture of EBV-infected B-cells with T-cells.
d Against autologous and allogeneic LCLs.


Cell-mediated Immune Reactions in Three Patients with Malignant Lymphoproliferative Diseases in Remission and Abnormally High Epstein-Barr Virus Antibody Titers

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