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Analysis of Epstein–Barr Virus-Specific and Non-Specific Immune Functions in a Patient During the Development of a Non-Hodgkin's Lymphoma

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Abstract—*A 57-year-old woman who presented with a reactive non-malignant lymphadenopathy was observed subsequently during the development of a nodular centroblastic non-Hodgkin's B-cell lymphoma. The Epstein–Barr virus (EBV)-specific antibody profile and EBV-specific and non-specific cell-mediated immune functions were determined at first presentation, and at various times during progression, in order to determine whether EBV was causally involved in the lymphoma and to assess in general the patient's cell-mediated immune function.*

At presentation, an immunodeficient status was suggested by an EBV-specific antibody profile indicative of an activated persistent infection; high antibody titers to viral capsid antigen (VCA) and early antigens (EA), but a low level of antibodies to EBV nuclear antigen (EBNA) confirmed by lack of leukocyte migration inhibition in response to EBNA (LMI-EBNA). The number of positive cells reactive with OKIa1 monoclonal antibody was significantly depressed, as was also the natural and interferon-activated killing (NK-IAK).

After emergence of the lymphoma, NK–IAK reactivity and spontaneous lymphocyte DNA synthesis augmented in parallel with an increase in the frequency of Leu-7$^+$ blood lymphocytes. The EBV-specific cell-mediated response, reflected by the outgrowth inhibition (OI) test was abolished in parallel with a decrease in the frequency of OKT3- and OKT4-positive lymphocytes.

INTRODUCTION

EPSTEIN–BARR virus (EBV) transforms B-lymphocytes *in vitro* [1, 2], is oncogenic in experimentally infected sub-human primates [3, 4], causes infectious mononucleosis (IM) [5], and is associated with two human malignancies, Burkitt's lymphoma (BL) [6] and nasopharyngeal carcinoma (NPC) [7]. Non-symptomatic infection with the virus is widespread in all human populations. Infected individuals carry the virus in their lymphoid tissues for life [8]. Considerable attention has been devoted to the identification of factors that maintain the virus–host balance, in particular the role of the host's immune responses.

Both primary and persistent EBV infections may take an unusual course in immunologically-compromised individuals. Depending on the immune defect, the clinical, hematological and serological responses may differ from those of immunologically-competent individuals [9]. The most frequently recorded change is an increase in the antibody titers against antigens associated with the viral replicative cycle, VCA and EA. Antibodies to EBNA show a contrasting behavior; they tend to decrease and are often undetectable. While the mechanism(s) of these changes are obscure, the dissociation between anti-EA/VCA and anti-EBNA titers suggests that different processes are responsible for restricting virus production and for controlling the proliferation of transformed EBNA-positive cells.

Immunologically-compromised EBV-infected patients fall into two categories: those who cannot, and those who can, restrict the proliferation of virally-transformed B-lymphocytes [10]. The first category includes patients with lymphoproliferative diseases after organ transplantation, and the familial X-linked lymphoproliferative syndrome. The second category includes some Hodgkin's and non-Hodgkin's lymphoma patients with a high EBV-load and an impaired cellular immune response. They show no enhanced risk of developing EBV-

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carrying lymphoproliferative malignancies. We have recently studied a series of patients belonging to the second category. They had a high frequency of $OKT8^+$ blood T-lymphocytes. Some showed an impaired capacity to control the *in vitro* proliferation of autologous EBV-transformed (EBNA-positive) cells in the outgrowth inhibition (OI) assay and a deficient production of leukocyte migration inhibitory factor (LMI) in response to EBNA [11, 12].

An opportunity arose to study a patient presenting with a reactive, non-malignant lymphadenopathy and high antibody titers to VCA and EA but a relatively low level of anti-EBNA. The patient was observed clinically during fifteen months. She developed a malignant non-Hodgkin's lymphoma of B-cell type during the fourteenth month of the observation period. We have examined her serology and EBV-specific and non-specific cellular immune responses at the stage of her benign lymphadenopathy, and at different times during the progression towards a malignant lymphoma, in order to assess a possible ABV etiology and determine in general the patient's cell-mediated immune functions.

PATIENT, MATERIAL AND METHODS

Case report

A 57-year-old woman was admitted to the hospital in July 1983 due to enlarged lymph nodes at her right side of the neck, left axilla and right groin which had persisted for three months. She had no symptoms except for tiredness.

Laboratory tests: ESR 11 mm/hr, Hb 141 g/l, WBC 7.0×10^9 with abnormal differential count, platelets 175×10^9/l, normal liver function tests, normal serum electrophoresis including immunoglobulin concentrations. Normal chest X-ray. No palpable splenomegaly. A lymph node was surgically removed. The histopathological diagnosis was a reactive non-malignant lymphadenitis (Table 1).

During Autumn 1983, and Spring 1984, the lymph node enlargement gradually decreased and her condition improved. However, an enlarged lymph node persisted in her left groin. A new lymph node biopsy was performed in May 1984, raising the suspicion of a malignant lymphoma (Table 1).

In the Summer of 1984 her general condition deteriorated with increased fatigue and loss of weight. A generalized lymphadenopathy developed. She received no treatment until then. Another lymph node was removed and the diagnosis of a nodular centroblastic non-Hodgkin's lymphoma of B-cell type, stage III B was established. She was treated with cyclophosphamide, doxorubicin, vincristine, prednisolone (CHOP)-methotrexate, leading to complete remission after three courses.

Table 1. Histopathological diagnosis of surgically-removed lymph nodes at three different times

July 1983	Reactive lymph node changes with angioimmunoblastic and nodular proliferation of immunoblasts. T-lymphocytes dominated with an increase in polyclonal B-cells. The morphology was inconclusive but probably a reactive lymphadenitis.
April 1984 (I)*	The cell morphology was highly variable. A progression has occurred since July 1983 with large confluent areas of centroblasts and immunoblasts. Immunohistochemistry showed a normal distribution of T- and B-cells but with a slight dominance of λ bearing lymphocytes. However, λ^+ lymphocytes engaged the lymph node only partly and monoclonality was not confirmed. Follicular-center derived centroblastic lymphoma was suspected.
September 1984 (III)*	Cell morphology was dominated by centroblasts with a nodular growth pattern. Immunohistochemical analysis revealed a dominance of monoclonal B-cells with the surface immunogobulin phenotype λ^+, μ^+, d^+. The histopathological diagnosis was nodular centroblastic non-Hodgkin's lymphoma.

*The battery of tests for EBV-specific and non-specific immune responses are indicated chronologically by Roman numbers.

EBV-specific antibody tests

IgA, IgG and IgM antibodies to VCA and to the D and R components of the EA complex were titrated by indirect immunofluorescence as described [13, 14]. Antibodies to EBNA were measured by anti-complement immunofluorescence [15].

Nucleic acid hybridization

Cellular DNA was prepared from the biopsy specimen by standard procedures [16]. The number of EBV genome equivalents was determined by the cRNA/DNA filter hybridization method with ^{32}P-labeled EBV cRNA prepared *in vitro* by transcription of EBV DNA, as a probe [17]. As a positive control the EBV-positive cell line Raji was used, while the EBV-negative cell line U-698 served as a negative control.

NON-EBV SPECIFIC CELLULAR IMMUNE REACTIONS

Surface markers

The following monoclonal antibodies were used to assess the marker distribution in the lymphocyte

populations: OKT3 (pan T), OKT4 (inducer/helper), OKT8 (suppressor/cytotoxic), OKIa1 (anti-Ia), (Ortho Pharmaceutical Rarithan, New Jersey 08869, U.S.A.) [18–22], and Leu-7 (Becton-Dickinson, Mountain View, CA, U.S.A.) [23].

Surface marker expression was assessed by indirect immunofluorescence [11, 12].

Spontaneous and mitogen induced DNA synthesis

Purified venous blood lymphocytes from the patient and healthy donors were tested for spontaneous, mitogen (PWM-GIBCO Berkley, CA; Con-A, Pharmacia Fine Chemicals, Uppsala, Sweden) and antigen (PPD, State Serum Institute, Copenhagen, Denmark) induced DNA synthesis as previously described [24].

Natural killing (NK) and interferon-activated killing (IAK)

Short-term (4 hr) ^{51}Cr release assays were performed as described [25]. Two target cells were used: the human erythroleukemia line K562, highly sensitive to unmanipulated NK cells, and the Burkitt's lymphoma line Daudi, relatively insensitive to NK but highly sensitive to interferon-activated killer (IAK) cells [26]. For interferon treatment, human leukocyte interferon (α-IFN) was used, derived from Namalwa cells afer induction with Sendai virus [27].

EBV-SPECIFIC CELLULAR IMMUNE RESPONSES

Outgrowth inhibition assay (OI)

Detection of outgrowth inhibition activity in EBV-infected peripheral blood lymphocytes from the patient was carried out as previously described [11, 12, 28].

EBV-specific leukocyte migration inhibition (LMI)

Cell extracts were prepared; direct and indirect LMI were performed as described [28, 29, 30]. The two techniques gave comparable results [31, 32].

The mean migration area was computed from triplicate sets; the migration inhibition index (MI) from the mean of the test (M_t) and of the control (M_c) samples was computed by the formula $MI = M_t/M_c$. An MI of < 0.80 was regarded as significant inhibition as in earlier studies [30, 33].

RESULTS

EBV serology

At admission the patient had markedly elevated IgG antibody titers to VCA and the D and R components of the EA complex but a relatively low level of antibodies to EBNA (Table 2). Also IgA antibodies to VCA and EA were recorded. Except for minor fluctuation in antibody titers, the serological profile remained constant during and after the development of a histopathologically-verified lymphoma (Tables 1 and 2).

Nucleic acid hybridization

cRNA/DNA hybridization of lymph node biopsies obtained during the non-malignant and the malignant phase (April 1984 and September 1984) showed the presence of less than 0.5 EBV genomes per 5 μg of bioptic material, i.e. EBV–DNA negative (data not presented).

Surface markers

In the first test (I in Table 3) corresponding to the diagnosis of reactive lymphadenitis the total lymphocyte count was low but within the normal range. After the diagnosis of malignant lymphoma the patient was lymphopenic (1100 cells/mm^3) (Test III).

The number of OKT3- and OKT4-positive lymphocytes decreased with time. In contrast the number of OKT8$^+$ lymphocytes remained relatively constant and was not significantly different from healthy donors. The percentage of OKT8$^+$ cells was increased. The OKT4/OKT8 ratio was normal (1.6) at Test I, but low at Tests II and III. The number and percentage of OKIa1$^+$ cells were significantly lower than in healthy individuals and remained constant during the period of the study. An increase in Leu-7$^+$ cells was observed at Test III.

Non-EBV-specific functional tests

Natural killing and interferon activated killing (NK-IAK). Natural cytotoxic activity against K562 and Daudi cell lines was lower during the pre-lymphoma stage (Tests I and II) than in healthy donors. This activity could be boosted by *a*-interferon (α-IFN) treatment, although it did not reach the level of healthy controls, tested in parallel (Table 4).

Subsequent to the diagnosis of the lymphoma, NK and IAK reactivities against K562 and Daudi cells were high compared to the pre-lymphoma stage. This increase was accompanied by a rise in the frequency of Leu-7$^+$ cells (Table 3).

DNA synthesis. The patient's lymphocytes showed a high spontaneous rate of DNA synthesis increasing progressively with time (Fig 1c). After stimulation with Con-A, PWM and PPD DNA synthesis was normal (data not shown).

EBV-specific immune responses

Outgrowth inhibition (OI). In Test I the blood lymphocytes inhibited the growth of EBV transformed autologous B lymphocytes, as in normal EBV-seropositive donors (Table 4). In Test II

Table 2. EBV serology and lymph node histology

Date	VCA IgA	VCA IgG	EA D IgA	EA D IgG	EA R IgA	EA R IgG	EBNA	Histology
8306	80	5120	< 10	80	10	640	20	Reactive lymphadenitis.
8404 (I)*	80	2560	< 10	80	10	320	20	A slight dominance of λ^+ lymphocytes. Monoclonality was not proven but there was a suspicion of malignant lymphoma.
8406 (II)	80	2560	< 10	80	10	320	20	No biopsy performed.
8409 (III)	80	5120	< 10	80	10	320	20	Nodular centroblastic lymphoma.
EBV pos. healthy donors†	< 10	104± 95	< 10	15± 28	< 10	15± 26	30± 15	

*See explanation in Table 1.
†Geometrical mean and S.D. of 20 healthy EBV-positive donors.

Table 3. Lymphocyte populations

Date	Total	OKT3	OKT4	OKT8	T4/T8	OKIal	Leu-7
8404 (I)	1680†	82* (1380)	43 (722)	27 (453)	1.6	4 (67)	17 (285)
8406 (II)	1300	81 (1053)	23 (300)	32 (416)	0.72	5 (65)	15 (195)
8409 (III)	1100	57 (627)	39 (429)	35 (385)	1.1	7 (77)	34 (374)
Healthy donors ($n = 20$)‡	(2500±178)	75±7‡ (1800±111)	43±5 (1075±78)	21±3 (525±44)	2.1±0.3	11±2 (220±10)	15±4 (277±25)

*Percentage of cells.
†Cells/mm^3.
Mean ± S.E., adult healthy donors between 20 and 60 years of age; 9 women, 11 men.

there was still measurable activity but a higher cell concentration was required for complete inhibition of B-cell proliferation (0.5×10^6).

In Test III the lymphocytes had completely lost their OI activity and became similar to an EBV-seronegative donor.

Leucocyte migration inhibition (LMI). At Test I (Table 4) EBNA reactivity was negative, i.e. no leucocyte migration inhibitory factors were released after cultivating the leucocytes in the presence of EBNA-containing cell extracts. Lymphokine production was induced by stimulation with EA/VCA antigen-containing cell extracts. This differs from the pattern of reactivity in normal EBV seropositive donors and resembles the findings in acute mononucleosis and certain forms of immunodeficiency. This pattern of reactivity was maintained throughout the observation period. It is relevant to notice that reactivity to VCA/EA antigen-containing extracts decreased during the observation period and by the time of lymphoma development, it was approaching a negative reaction (< 0.80).

Relation between lymphocyte subpopulations and cellular immune responses

The percentage of $OKT3^+$ and $OKT4^+$ cells decreased together with OI activity (Fig. 1a). The number of Leu-7^+ cells correlated with an increase of IAK activity against Daudi (Fig. 1b). The same was found for NK–IAK activity against K562 (not shown). EBV-non-specific immune parameters, (DNA synthesis, IAK activity against Daudi and percentage of Leu-7^+ cells), increased in parallel; while the EBV-specific immune responses (OI and LMI–EBNA), showed the opposite trend.

DISCUSSION

About 2% of HD and NHL patients develop unusually high IgG, and often also IgA antibody titers to VCA and to the D and/or R components of the EA complex. In contrast, antibodies to EBNA remain at low levels or become non-detectable [12, 29, 34, 35]. A dissociation between antibody production to antigens associated with viral cycle (VCA, EA) and antigens of latently EBV-infected, immortalized B-cells (EBNA), is seen also in pri-

Table 4. EBV non-specific and EBV-specific immune responses

Date	NK*		IAK*		OI†	LMI‡	
	K562	Daudi	K562	Daudi		EBNA	VCA/EA
8404 (I)	20	13	30	26	<0.25	0.86	0.61
Control§	35	15	65	50			
8406 (II)	7	5	20	20	0.5	nd	nd
Control	40	13	66	60			
8409 (III)	39	26	58	50	>2.0	0.99	0.78
Control	34	24	65	56			
Healthy donors‖	35±11	27±14	42±15	43±11	>2.0¶	0.90±0.01¶	0.83±0.01¶
(n = 25)					<0.25**	0.57±0.02**	0.61±0.01**

* Natural killer cells (NK) and aIFN inactivated killer cells (IAK) activity: percentage of ^{51}Cr release at 25 : 1 effector target ratio. K562 and Daudi are the target cells used.

† Outgrowth inhibition (OI); number of cells × 10^6 necessary to induce 50% OI. 2.0 × 10^6 cells corresponds to lack of inhibition, i.e. a negative response <0.25 × 10^6 means an effective inhibition of EBV-transformed B-cells, i.e. a positive response.

‡ Leucocyte migration inhibition (LMI) is expressed as migration index: MI = M_t/M_c, when M_t = migration area in presence of antigen and M_c = control migration (without antigen).

§ Age-matched healthy individuals in the same test.

‖ Mean and S.E. of 25 healthy donors tested at different occasions and used as a normal reference; between 20–60 years old, 11 women and 14 men.

¶ EBV-negative control donors.

** EBV-positive control donors.

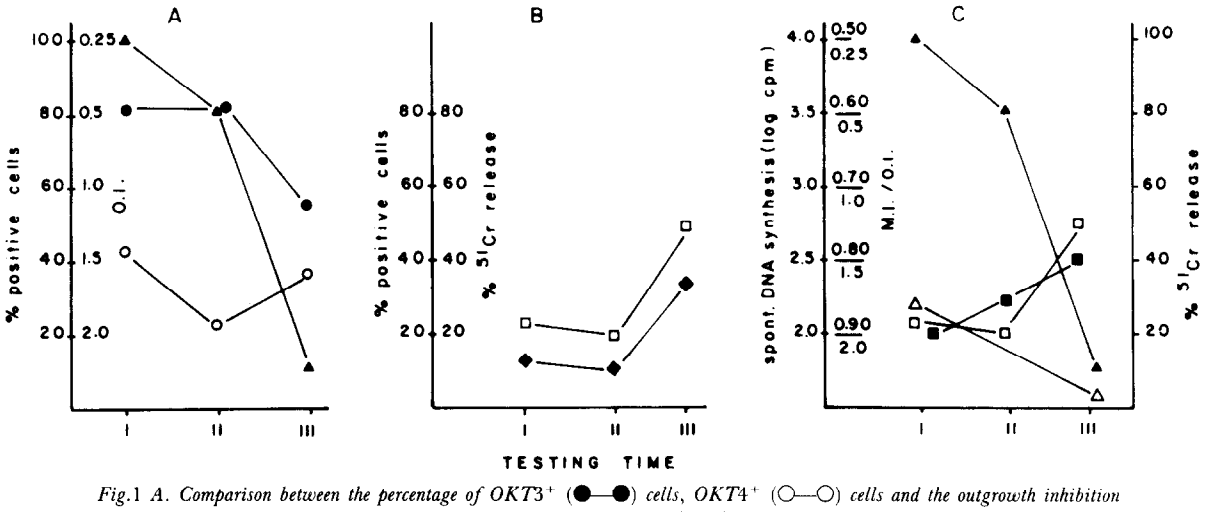

Fig.1 A. Comparison between the percentage of OKT3⁺ (●—●) cells, OKT4⁺ (○—○) cells and the outgrowth inhibition activity (▲—▲). B. Comparison between the percentage of Leu-7⁺ (◆—◆) cells and the percentage of interferon-activated killing activity against Daudi (IAK-Daudi) cells (□—□). C. Comparison between EBV non-specific and EBV-specific cellular immune responses: (■—■) spontaneous DNA synthesis; (□—□) interferon-activated killing against Daudi; (▲—▲) OI; (△—△) LMI-EBNA.

mary EBV infections, whether accompanied by symptoms of infectious mononucleosis (IM) or not. Antibodies to VCA and D reach peak titers long before the emergence of anti-EBNA [14]. Even the late anti-R responses seen in IM patients precede anti-EBNA as a rule [36].

Some HD patients develop excessively high EBV antibody titers which are maintained during several years. NHL patients have often high antibody titers already at the time of diagnosis or within a few months thereafter [12]. In the present case high antibody titers to VCA and an anti-EA-R titer, than exceeded the anti-D level, were detected well before the diagnosis of NHL.

The 57-year-old female patient presented initially with a reactive, non-malignant lymphadenopathy. A second biopsy taken 10 months later raised the suspicion of a malignant lymphoma which was definitely diagnosed after a further 5 months. The EBV-specific antibody profile remained essentially constant during the 15 months and for 10 further months (data not shown). Thus, the emerging

malignant lymphoma did not affect the EBV antibody titers. This is not surprising because nucleic acid hybridization failed to reveal the presence of detectable EBV-genomes in the lymph node biopsies that contained lymphoma tissue. While the patient was immunodefective by several criteria, as already stressed, her impairment was apparently insufficient to permit proliferation of EBV-carrying lymphoid clones. This is similar to our previous experience on HD and NHL patients with impairment of EBV specific and non-specific immune responses, but no EBV-carrying lymphoproliferative disease [12]. Since the potential immunogenicity of the developing EBV-negative lymphoma is not known, the possible pathogenetic significance of the immune impairment for lymphoma development in these patients remains unknown.

The low NK and IAK activity seen during the pre-lymphoma stage reminds one of the situation observed in Chediak–Higashi patients, where high levels of anti-VCA and EA-R antibody titers were found in parallel with low NK and ADCC reactivity [37]. Both activities augmented in the lymphoma stage, concomitantly with an increase in Leu-7$^+$ cells. This parallels our previous observations on HD and NHL patients with high VCA titers [12]. There was also a progressive increase in spontaneous DNA synthesis. This probably corresponds to the large, activated *in vivo* lymphocytes. They include mainly large basophilic cells within the T- and non-T/non-B-lymphocyte populations that can mediate NK–IAK activity [38, 39].

The progression of the disease was accompanied by an increasing lymphopenia mainly due to the lack of OKT3$^+$, OKT4$^+$ and OKIa1$^+$ cells. It has been suggested that a depressed expression of Ia antigens may be paralleled by defective antibody and cell-mediated immune responses [40]. The number of OKT8$^+$ lymphocytes remained constant and within the normal range. Consequently the OKT4/OKT8 ratio decreased, leading to a relative increase in suppressor cells in the lymphoma stage.

In a previous study the majority of HD and NHL patients with high titers against VCA had a low OKT4/OKT8 ratio [12]. There were no analogous changes in patients with low antibody titers. It was therefore postulated that suppressor T-cells may participate in the host response, with high antiviral EBV titers or, alternatively increased activation of the viral cycle, as a consequence.

The decrease in the number of total T- and helper T-cells was related to the disappearance of outgrowth inhibition (OI) activity. This EBV-specific immune response is known to be mediated by T-lymphocytes [41], and it has been shown that suppressor T-cells are responsible for the inhibition of EBV-transformed autologous B-cells *in vitro* [42].

The question arises whether the impaired T-cell reactivity of the patient in the prelymphoma stage, as reflected by her EBV reactivity pattern and her immunological profile, played any role in permitting the outgrowth of her B-cell lymphoma. It cannot be excluded, however, that the lymphoma cells were potentially antigenic, in spite of their EBV-negativity. In this connection it is of interest that the excess lymphoma appearing in AIDS or pre-AIDS patients includes both EBV negative and positive B-cell lymphomas [43].

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