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Advances in Brief

Rapid Proliferation of B Cells from Adenoids in Response to Epstein-Barr Virus Infection[1]

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Abstract

EBV is a human tumor virus that is associated with different types of tumors. A unique feature of EBV is its capability to infect and immortalize human B cells both *in vivo* and *in vitro*. In cell culture, this progress is termed immortalization and infected B cells grow out to permanent, so-called lymphoblastoid cell lines. During our experiments, we observed that B lymphocytes derived from adenoids are infected efficiently by EBV and proliferate much more rapidly than any other known type of B cell. High concentrations of adhesion molecules and of CD21, the EBV receptor, present on these cells may account for this phenomenon. Adenoid B cells may therefore represent a particular subpopulation of preactivated B lymphocytes that can greatly simplify and enhance the production of lymphoblastoid cell lines for, *e.g.*, antigen-presenting cells for gene therapeutic approaches and similar applications.

Introduction

Hyperplasia of the pharyngeal tonsil, the so-called adenoids, are common in children. Adenoids are part of the Waldeyer's tonsillar ring and are derived from lymphatic tissue. Histologically, adenoids are characterized by the presence of chronic inflammation and varying degrees of fibrosis. Clinically, larger adenoid affect the upper airway by nasopharyngeal obstruction, causing complications in breathing and swallowing. Children concerned are prone to repeated infections and impaired development. Due to the mechanical obstruction of the eustachian tube, a conductive deafness may also develop. To prevent retardation in speech development and to improve the general health, adenoids ought to be resected in early years. Despite the numerous patients concerned, the etiology of adenoids is still unknown. Several theories have been proposed that are based mainly on allergic or infectious agents. Latent EBV infections have only been described in the adenoids of patients suffering from posttransplant complications (1), and adenovirus type 2 DNA has been detected, although without any sign of a true infection (2).

EBV is a human tumor virus that is associated with different kinds of tumors. Viral DNA can be isolated from most Burkitt's lymphoma and nasopharyngeal carcinoma (3, 4). Many cases of Hodgkin's disease and T-cell lymphoma have also been found positive for EBV (5). Primary infection with the virus usually takes place during early childhood and is clinically unapparent. However, in certain cases, primary infections with EBV can cause infectious mononucleosis with up to 10% EBV-infected lymphocytes in the peripheral blood. Target cells for primary infection are thought to be epithelial cells of the nasopharyngeal region. It is believed that these cells support only the lytic cycle of EBV and undergo lysis upon viral infection due to the production of virus particles. In contrast, B lymphocytes are usually infected latently and are nonproductive (6).

EBV is the only known human virus that is capable of inducing and maintaining proliferation of infected human B lymphocytes both *in vivo* and *in vitro*. For this reason, EBV is often used as an *in vitro* model for cell immortalization and related investigations. For the generation of permanent, so-called LCLs.[3] B lymphocytes are incubated with infectious EBV particles present in the supernatant of producer cell lines. Infected and immortalized cells start dividing after a certain time of infection and proliferate indefinitely *in vitro*. This way, LCLs from any human donor can be established quite easily and are used as antigen-presenting cells for the generation of antigen-specific cytotoxic T-cell lines or in adoptive immunotransfer approaches (7, 8). However, the immortalization process of these cells is slow; it takes 2 to several weeks until the cells start proliferating.

During *in vitro* infections with EBV of primary B lymphocytes derived from routine adenoidectomies, we observed that these cells are infected readily and start proliferating much faster than B lymphocytes from tonsils and peripheral blood, which are traditionally used for this kind of investigation. Depending on the donor, typical clones of immortalized B cells that give rise to multiple permanent LCLs can be detected as early as 2 days after infection. These adenoid-derived B lymphocytes seem to consist of a very particular subpopulation that is especially susceptible to EBV-delivered growth stimuli. The molecular background for this phenomenon, however, remains to be elucidated.

Materials and Methods

Cell Preparation and Virus Infection. Primary human B lymphocytes were isolated from routine adenoidectomies or tonsillectomies or were isolated from heparinized blood of healthy donors. T cells were depleted by rosetting with whole sheep blood as described.[4] For the analysis of adenoid and tonsillar cell populations, T-cell depletion was omitted. After centrifugation on a Ficoll cushion at 560 × *g*, cells were washed and resuspended in cell culture medium. The preparations were analyzed by FACS and were found to be >95% positive for the pan-B marker CD19 (DAKO, Hamburg, Germany). For virus infection, B lymphocytes were incubated with cell-free supernatants from the producer cell line B95.8 (9). Cell-free B95.8 supernatant was generated by pelleting densely grown B95.8 cells at 350 × *g* and passing the supernatant through a 0.45 μm filter (Nalgene, Hereford). VFS was obtained by ultracentrifugation of cell-free B95.8 supernatant at 30,000 × *g* for 4 h. In contrast to the cell-free supernatants, no viral DNA was detectable in VFS by PCR analysis (data not shown).

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[3] The abbreviations used are: LCL, lymphoblastoid cell line; FACS, fluorescence-activated cell sorting; VFS, virus-free supernatant; IL, interleukin; ICAM, intercellular adhesion molecule; LFA, leukotactic factor activity.

[4] R. Zeidler, G. Eissner, P. Meissner, S. Uebel, R. Tampé, S. Lazis, and W. Hammerschmidt, Downregulation of TAP1 in B lymphocytes by cellular and Epstein-Barr Virus encoded IL-10, submitted for publication.

[^{3}H]Thymidine Incorporation. For the determination of DNA synthesis, 10^4 primary B lymphocytes were seeded in a volume of 100 μl/well of cell culture medium or supernatant of the marmoset cell line B95.8 (9). All supernatants were supplemented with 10% FCS. Cells were kept at 37°C for up to 1 week. [^{3}H]Thymidine (0.5 μCi) was added for 4 h, and incorporation was measured in harvested cells as described (10).

RNA Preparations and RT-PCR. Total cellular RNA was prepared as described (11). Total RNAs from primary B lymphocytes were isolated after 48 h of incubation with native B95.8 supernatant or VFS from the same cells. The RNA preparations were treated with RNase-free DNase (Boehringer Mannheim, Mannheim, Germany) for 30 min at 37°C. After inactivation of the enzyme, 1 μg of RNA was reverse transcribed (Superscript Plus; Life Technologies, Inc., Gaithersburg, MD) with an oligodeoxythymidylic acid primer for 60 min at 37°C. PCR with 1/10 of the volume (2 μl) was performed in the presence of 1.5 mM $MgCl_2$, 100 pmol of each primer, a 0.2 mM final concentration of each dNTP, and 0.5 μl of Goldstar Taq polymerase (Eurogentec, Seraing, Belgium) in a final volume of 50 μl in a Perkin-Elmer Corp. thermal cycler. Cycling conditions (30 cycles) were 94°C for 45 s, 60°C for 60 s, and 72°C for 60 s. Amplified bands were analyzed by electrophoresis through a 1.5% agarose gel and ethidium bromide staining. Primers used were the following (listed in the 5′ to 3′ direction): CCTTAGGCTCACCCATTTCAACC and TCCTTCAAGCAGGAGAAAAGAGAG for IFN-α; AGCTCTGCATCGTTTTGGGTTC and CAAATATTGCAGGCAGGACAACC for IFN-γ; CAAGAATCCCAAACTCACCAGG and CAATGGTTGCTGTCTCATCAGC for IL-2; CGGACACAAGTGCGATATCACC and CCAACGTACTCTGGTTGGCTTCC for IL-4; CACACAGACAGCCACTCACCTC and CTCAGGCTGGACTGCAGGAAC for IL-6; TTTCCGCTCTGAGTCCCTTGAG and TGGAGCCATTGAAGGTTCTGG for IL-12/p40; TCATTGCTCTCACTTGCCTTGG and GTTTCAGTTGAACCGTCCCTCG for IL-13; CAGATCCTGTCCAAGCTGCGGC and GAATGGTGGCCAGGTCACCTCGGC for transforming growth factor β; GAGCTGAGAGATAACCAGCTGGTG and CAGATAGATGGGCTCATACCAGGG for tumor necrosis factor α; and AATTCCATGGCACCGTCAAG and GCCTGCTTCACCACCTTCTT for glyceraldehyde-3-phosphate dehydrogenase, which served as an internal efficiency control.

FACS Analysis. Mononuclear cells were isolated from heparinized blood or from routine ectomies of tonsils and adenoidal tissues by Ficoll density centrifugation as described above. Flow cytometry was assessed by indirect immunofluorescence using the FACS flow cytometer and the CellQuest analysis program (Becton Dickinson, Heidelberg, Germany). Cells were incubated

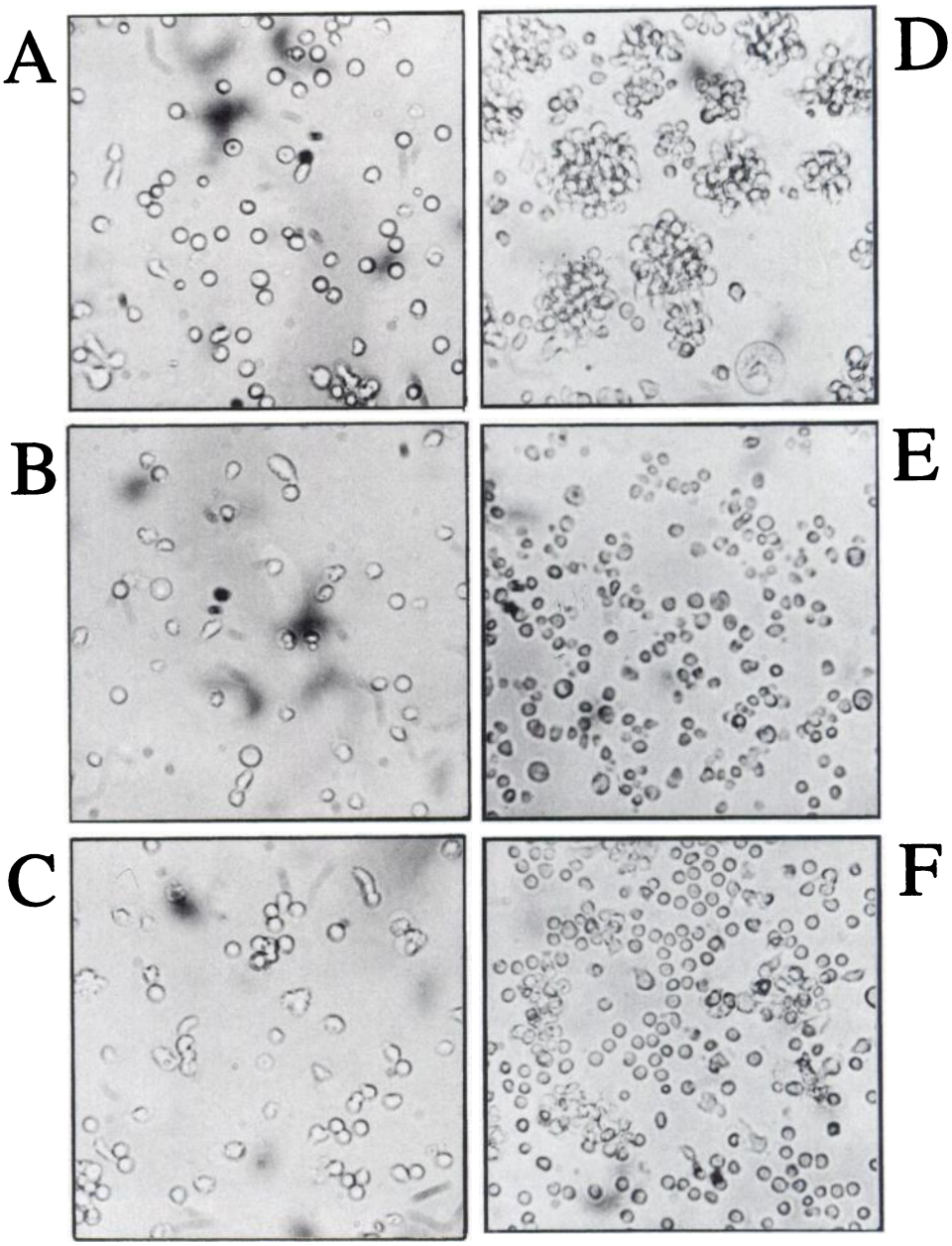

Fig. 1. B lymphocytes from adenoids, tonsils, and peripheral blood were incubated with cell culture medium (*A*, *B*, and *C*, respectively) or with the EBV-containing supernatant of the cell line B95.8 (*D*, *E*, and *F*, respectively). As soon as 2 days after EBV infection, B lymphocytes from adenoids grew in typical clusters (*A*). Endogenous EBV infection cannot account for proliferation of these cells, because cells incubated with a control supernatant (cell culture medium) showed no sign of proliferation (*D*). B cells from tonsils and peripheral blood, which were infected with the identical B95.8 supernatant (*B* and *C*) still display the single-cell appearance as with cell culture medium (*E* and *F*).

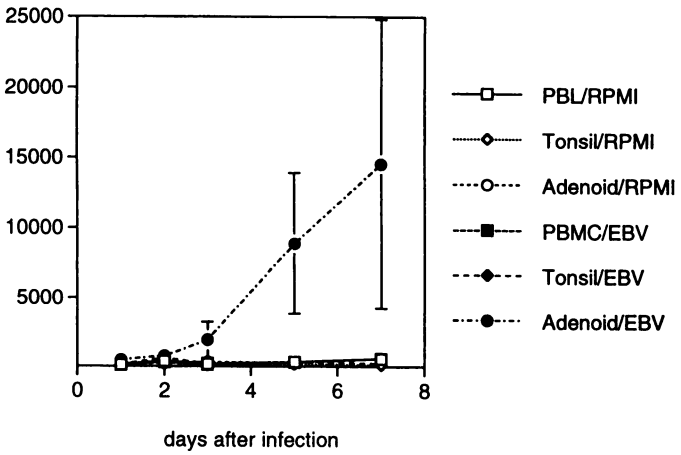

Fig. 2. [^{3}H]Thymidine assay to measure the proliferation of primary B lymphocytes in response to EBV infection. Cells were isolated from three different donors, and each preparation was seeded in triplets. Values shown are means; *bars*, SD. Cells (10^4) were seeded in 100 μl of RPMI or B95.8 supernatant, and [^{3}H]thymidine incorporation was determined as described in "Materials and Methods." Proliferation of B lymphocytes isolated from adenoids and incubated in EBV-free cell culture medium is not detectable within the time point indicated. *PBL*, peripheral blood lymphocyte; *PBMC*, peripheral blood mononuclear cell.

with appropriate primary antibodies for 60 min on ice (DAKO, Hamburg, Germany; Dianova, Hamburg, Germany). After a single washing, cells were incubated with a secondary antimouse IgG-FITC-conjugated antibody (DAKO, Hamburg, Germany) for 45 min. Cells were analyzed after a final washing.

Results

B Lymphocytes from Adenoids Show Typical Homotypic Aggregation. EBV-immortalized B lymphocytes grow in typical clusters that can be easily detected macroscopically. This homotypic aggregation is due to the elevated expression of adhesion molecules ICAM-1, LFA-1, and LFA-3 (12, 13). As shown in Fig. 1, B lymphocytes from adenoids proliferate in clusters as soon as about 2 days after infection, whereas those derived from tonsils and peripheral blood are still in the single-cell state without any detectable cell divisions. Proliferation was also confirmed by cell counting (data not shown). RT-PCR and [^{3}H]thymidine assays demonstrate clearly that adenoid B lymphocytes have not been EBV infected prior to *in vitro* infections with B95.8. These cells, when incubated with EBV-free cell culture medium, do not proliferate (see Fig. 2), and viral gene expression could not be detected (data not shown). Sporadic spontaneous proliferation of cells after incubation with cell culture medium was observed occasionally with cells from tonsils, adenoids, or peripheral blood as expected from several donors not depending on the tissue which is due to an *in vivo* EBV infection. However, these contaminations affected only a minimal proportion of cells and did not became apparent before 1 week of *in vitro* cultivation. Therefore, the preinfected cell population cannot account for the tremendous differences in proliferation.

B Lymphocytes from Adenoids Start Proliferating Much Faster than Those Derived from Tonsils and Peripheral Blood. To investigate the proliferative behavior of B lymphocytes derived from different donors and organs, cells from the peripheral blood, freshly ectomized tonsils, and freshly taken adenoids were compared. To this end, B lymphocytes were isolated from the different origins and were infected with supernatants of the marmoset cell line B95.8, which contains infectious EBV at about 10^6 immortalizing particles per ml. To determine the proliferation rate, 3[H]thymidine was added for 4 h, and its incorporation into the cellular DNA was measured as described in "Materials and Methods." As shown in Fig. 2, cells from adenoids start growing as soon as 2 days after infection. Cell division is obvious by the detection of mitotic cells and the appearance of cell clusters that are typical for proliferating LCLs (see below). In comparison, growth of B lymphocytes from tonsils and peripheral blood was never observed earlier than 7 days after infection, as determined by 3[H]thymidine incorporation (Fig. 2).

Distribution of Cells from Adenoids and Tonsils Show No Significant Differences. To understand why B lymphocytes from adenoids start proliferating much faster in response to an EBV infection, we characterized the total cell populations present in the biopsies. To this end, we examined the surface antigenic pattern of the isolated cells by FACS analysis. No significant differences in lymphocytic markers could be detected for the investigated antigens (see Table 1). Cells present in preparations from tonsils and adenoids consist mainly of B lymphocytes (about 2/3 to 3/4), T lymphocytes (about 1/3), and a small proportion of macrophages and natural killer cells. About half of the cells displayed high concentrations of MHC class II molecules. The activation marker CD80 (B7.1) was present

Table 1 *FACS analysis of surface antigens on total cell populations isolated from adenoids and tonsils*

Proportions of cells (%) stained positive for different CDs are listed. No significant differences were detected concerning surface antigens and cell distribution between cells isolated from adenoids and tonsils. T lymphocytes, as judged by the detection of CD 2, 3, 4, and 8 are present at about 30% with more than 2/3 being of the CD4$^+$ helper subset. Machophages (CD14) and natural killer cells (CD56) form only about 1% each of the total cell population. B lymphocytes are present at about 65–75%.

	% Positive cells	
Surface antigen	Adenoids	Tonsils
CD2[a]	22–30	7–21
CD3	19–26	7–19
CD4	18–22	5–16
CD8	3.6–5.5	2.7–5.0
CD10	6–8	4–11
CD14	0.8–1.3	0.3–0.8
CD19	62–67	66–78
CD34	0.1–0.3	0.0–0.1
CD56	0.8–1.4	0.4–1.4
CD80	1.1–2.5	1.5–3.1
MHC class I	98–99.7	98.5–99.8
MHC class II	42–69	40–65
IgA	3.9–7.7	3.5–6.0

[a] CD, clusters of differentiation.

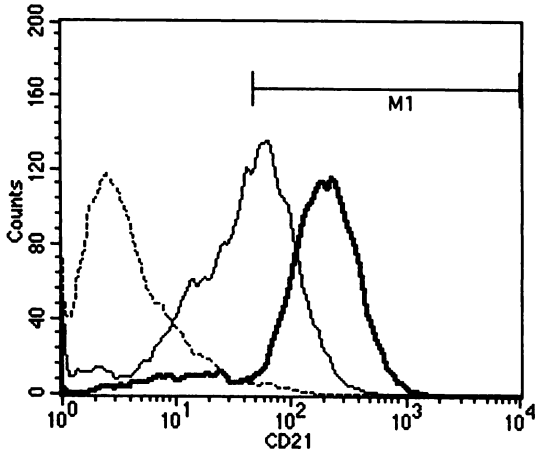

Fig. 3. Comparison of surface antigens concentrations by FACS analysis revealed a significantly higher density of the EBV receptor and complement receptor CD21 on B lymphocytes derived from adenoids compared to tonsillar B cells. Results are representative for four independent experiments. ·····, isotype control; ——, tonsillar B lymphocytes; ▬, B lymphocytes from adenoids. Mean fluorescence values are 3.5, 54, and 199, respectively.

Table 2 *Surface antigens on B lymphocytes from adenoids show elevated concentrations for adhesion molecules and activation markers*

Note the much higher concentration for the EBV receptor CD21 on B lymphocytes for adenoids. Concentrations of the B-cell marker CD19 were found to be almost identical on B cells derived from either type of tissue. Representative results of one out of four experiments are shown.

Surface antigen	Mean fluorescence	
	Adenoids	Tonsils
CD11a[a] (LFA-1)	27	7
CD21	199	58
CD40	408	62
CD44	1022	293
CD54 (ICAM-1)	73	21
CD58 (LFA-3)	59	12
CD102 (ICAM-2)	554	173

[a] CD, clusters of differentiation.

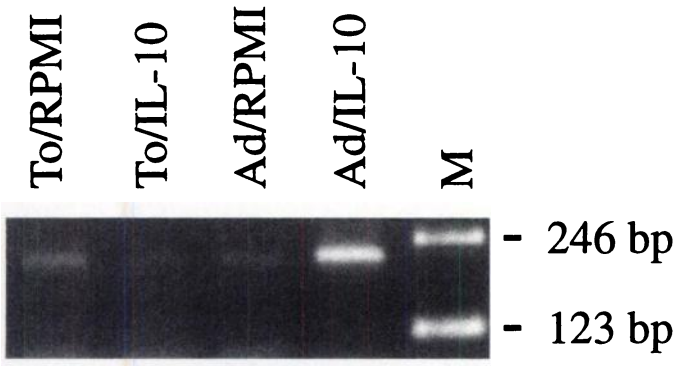

Fig. 4. B cells from adenoids, peripheral blood (not shown), and tonsils produce comparable amounts of IFN-α, as monitored by RT-PCR. However, the expression of IFN-α was elevated 2 days after EBV infection in B cells from adenoids, whereas those derived from tonsils and peripheral blood (not shown) showed reduced amounts of IFN-α mRNA, which is probably due to the viral IL-10 present in B95.8 supernatants.

only on a minor subpopulation (1–3%). In addition, no significant differences regarding the concentrations of CD19, MHC class I, and MHC class II were observed.

B Cells from Adenoids Are Preactivated. We next examined the B lymphocytes more intensely for activation markers. Our experiments revealed that B lymphocytes from adenoids display much higher concentrations of the complement receptor CD21, which is also known as the EBV receptor, on B lymphocytes (see Fig. 3). Thus, the elevated CD21 concentrations may account for the rapid EBV infection of cells derived from adenoids. Moreover, those cells show much higher concentrations for different activation markers (most noteworthy is the CD40 antigen) and adhesion molecules as shown in Table 2.

B Cells from Adenoids Express High Levels of IFN-α after EBV Infection. Our initial experiments indicated that B cells derived from adenoids differ from B cells isolated from peripheral blood or tonsils. Surprisingly, the overall composition of B cells from different organs was similar and could not account for the rapid proliferation of B lymphocytes from adenoids. This finding could mean that the B cells differ with regard to their activation status, which is also determined by the expression levels of IL-2, IL-4, IL-6, IL-12, and IL-13. However, B lymphocytes isolated from adenoid and tonsillar tissues express comparable levels of IL-2, IL-4, IL-12, IL-13, transforming growth factor β, tumor necrosis factor α, and IFN-α and IFN-γ, which were not altered within 2 days after EBV infection (data not shown). No expression of IL-6 was detected. The expression of both IFN-α and IFN-γ are high in tonsillar and peripheral B lymphocytes but are down-regulated after EBV infection, which is probably due to viral IL-10 present in B95.8 supernatants.[4] B lymphocytes from adenoids also express high levels of IFN-α and IFN-γ, and the level of IFN-γ mRNA is also repressed after EBV infection (data not shown). However, IFN-α mRNA is elevated in response to EBV infection (Fig. 4). Up-regulation of IFN-α mRNA after incubation with EBV-containing B95.8 supernatants was never observed with B lymphocytes from tonsils or peripheral blood.

Discussion

During our studies with EBV infections of primary human B lymphocytes, we observed that cells that have been isolated from freshly resected adenoids start to proliferate very quickly in response to an EBV infection. As soon as 2 days after infection, adenoid B lymphocytes grow in typical clusters and show considerable uptake of 3[H]thymidine. The 2-day period is much shorter than for B lymphocytes derived from tonsils or peripheral blood and to our knowledge has never been described before. To investigate this phenomenon, we performed additional FACS analysis to investigate the expression of different surface molecules. B lymphocytes from adenoids show, in comparison to those from tonsils, elevated concentrations for all antigens tested (see Table 2). The presumably most important difference seemed to be the significantly higher amounts of CD21 antigen, which is known as the EBV receptor and therefore may account for the efficient EBV infection of B cells from adenoids. Besides CD21, the presence of high amounts of CD40, which is essential for B-cell responses to thymus-dependent antigens, is a sign of the preactivated status of these B lymphocytes (14). High concentrations of adhesion molecules (ICAM-1 and -2; LFA-1 and -3) facilitate T-cell recognition of specific peptide-MHC class I and II complexes and may be indicative of a high "antigen-presenting activity" of B cells derived from adenoids (15). The preactivation of these B lymphocytes may be caused by a viral infection, albeit not EBV, because EBV DNA was not detected in the adenoid specimen.

The route of an EBV infection from the first contact with the host to the latent infection of B lymphocytes as well as cellular events during infection are far from understood. Our observation that B cells that reside in close vicinity to the nasopharyngeal epithelium are very responsive to EBV makes this region a good candidate for the place at which EBV first overcomes the barrier between epithelial and lymphoid cells. The higher amount of CD21 may be necessary for the infection itself, and a preactivated status may enable the infected cells to proliferate much more rapidly. The recently described differential display technique (16) has been successfully used for the isolation of differentially expressed genes (footnote 4; data not shown) and therefore may also be applicable for more detailed investigations on these particular types of B cells and the etiology of adenoids.

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