

Epstein-Barr Virus (EBV)-Containing Nasopharyngeal Carcinoma Cells Express the B-Cell Activation Antigen Blast2/CD23 and Low Levels of the EBV Receptor CR2

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Anaplastic nasopharyngeal carcinoma (NPC) cells invariably harbor the Epstein-Barr virus (EBV) genome, an association that is unique among human virus-associated cancers. Although EBV is able to replicate in epithelial cells, results with expression of the EBV receptor (complement receptor type 2 [CR2]; also called CD21) in normal and malignant epithelial cells are conflicting. We grew five different EBV-associated NPC tumors in nude mice, and by using a sensitive transcriptional assay, we detected a very weak transcription signal of the EBV receptor CR2 gene in these cells. This suggests that low levels of EBV receptor may be expressed by malignant epithelial nasopharyngeal cells. The gene coding for Blast2/CD23, a B-cell activation molecule induced by EBV, was transcribed in three of the transplanted NPC tumors. The soluble form of the Blast2/CD23 protein was also detected in medium taken from short-term cultures of the same NPC cell lines. In contrast to the lymphoid system, in which Blast2/CD23 expression is associated with EBV nuclear antigen (EBNA2) expression, no EBNA2 protein could be detected in these NPC epithelial cells. Our study represents the first demonstration of Blast2/CD23 expression in epithelial cells. As the soluble form of the Blast2/CD23 protein possesses growth factor activity associated with EBV-induced B-cell immortalization, these results suggest a possible role for this molecule in the pathogenesis of NPC.

Nasopharyngeal carcinoma (NPC) is seen worldwide, but it occurs with a very high incidence in Southern China, where it represents the most common tumor in males (for review, see reference 9). Two striking features of this cancer are the constant detection of Epstein-Barr virus (EBV) genome in NPC cells (8, 39) and the regular infiltration of the tumor by nonmalignant lymphocytes (16).

EBV is a B-lymphotropic virus, and therefore the invariable presence of EBV DNA in malignant epithelial NPC cells is puzzling. The B-cell receptor for EBV is a cellular glycoprotein of 145 kilodaltons (kDa), identical to the receptor for the C3d fraction of complement, complement receptor type 2 (CR2; also called CD21) (11, 12). CR2 is mainly expressed on B lymphocytes, although it is also present on follicular dendritic cells (22). A molecule sharing one epitope with the EBV receptor CR2 and recognized by three anti-CR2 monoclonal antibodies is also expressed on normal stratified pharyngeal and ectocervical epithelia in a cell differentiation-dependent manner (26, 34, 36).

No clear-cut results have been obtained concerning the expression of EBV receptor CR2 on NPC cells (13, 14). Studies have been hampered mainly by the lack of a long-

term culture method for NPC cells and because NPC tumors are heavily infiltrated with lymphoid cells.

Despite the presence of the EBV genome in NPC cells, its role in the malignant process is unknown. EBV is known to be a potent "immortalizing agent" capable of transforming human B lymphocytes to a state of continuous proliferation in vitro. A set of leukocyte markers are known to be up regulated in EBV-infected B cells, and these are probably involved in the immortalization process (6). These include not only the EBV receptor CR2, but also the 45-kDa leukocyte antigen Blast2/CD23, which is turned on in B cells following EBV infection (29, 30). Blast2/CD23 seems to be a pivotal molecule in EBV-induced cell proliferation since it is a labile protein transiently expressed on the cell surface and then processed and shed as a soluble form which possesses B-cell growth factor activity (28). As for the study of EBV receptor CR2, the analysis of Blast2/CD23 expression in NPC has been difficult due to lack of an efficient in vitro NPC culture system.

Through the transplantation of NPC tumors into nude mice, it was possible to obtain abundant homogeneous material without lymphoid contamination. We have been able to perform a sensitive transcriptional study, and we show that NPC cells express Blast2/CD23 but only weakly express the EBV receptor CR2.

MATERIALS AND METHODS

Nude mice and transplantable NPC tumors. Five human undifferentiated NPC tumors were serially transplanted in

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nude mice for more than 10 passages and designated C15, C17, C18, T1530 and T1915. The C15, C17, and C18 tumor cell lines were established at the Institut Gustave Roussy (Villejuif, France) from two patients from North Africa (C15 and C18) and one from Europe (C17) (4). The two other cell lines, T1530 and T1915, were established from Chinese patients by one of us (D.H.) at the Chinese University of Hong Kong. They were characterized by electron microscopy as epithelial cells by the presence of desmosomes and keratin filaments. In order to verify that the transplanted NPC tumors were of human origin, total cellular DNA was extracted from the five NPC tumors passaged in nude mice, from human cells, and from mice tissues. Each DNA (5 µg) was analyzed by dot blotting and hybridized with the human-specific *Alu* and with the murine-specific B1 probes. We were able to confirm that transplanted NPC tumors were composed mainly of human malignant materials (data not shown). The mouse cell infiltrates quantified with the B1 probe, while constant between the different NPC tumors, were nevertheless higher than the 1% of the cell population described previously (4). EBV nuclear antigen (EBNA) was detected by anticomplement immunofluorescence in all five tumor lines, but antigens of the replicative cycle, such as VCA, EA, and MA antigens, were not detectable (4).

RNA isolation and Northern (RNA) blot analysis. Total cellular RNA was prepared by the guanidium isothiocyanate-cesium chloride centrifugation method (7). From 30 to 40 µg of each RNA was size fractionated on a 1% (wt/vol) agarose gel containing 2.2 M formaldehyde and transferred in 20× SSC solution (20× SSC is 3 M NaCl, 0.3 M trisodium citrate · 2H₂O, pH 7) to GeneScreen Plus membrane (Du Pont) for hybridization. CR2, CD23, and GAPDH probes were prepared by random priming with [α -³²P]dCTP of the 1.6-kilobase (kb) *EcoRI* cDNA fragment from plasmid CR2 p1.6 (kindly provided by Janis Weis [33]), 1.7-kb *EcoRI* cDNA fragment from plasmid pFceR1 (kindly provided by Hiroshi Kikutani [18]), and total plasmid pRGAPDH-13 (kindly provided by Marc Piechaczyk, Université du Languedoc, Montpellier, France). Filters were prehybridized and hybridized overnight at 42°C in 0.05 M sodium phosphate buffer (pH 7)–1 M NaCl–50% (vol/vol) formamide–1% (wt/vol) sodium dodecyl sulfate (SDS)–100 µg of salmon sperm DNA per ml–100 µg of yeast tRNA per ml and then washed at 65°C for 1 h in 2× SSC containing 1% (wt/vol) SDS and exposed to Hyperfilm (Amersham) for autoradiography for 2 to 8 days. The level of CR2 mRNA in each cell line was compared with the glyceraldehyde phosphate dehydrogenase (GAPDH) mRNA level, which was assumed to be constant in cells of the various lines.

Plasmid construction and in vitro transcription. PG3Z/CR2 was constructed by subcloning the 450-base-pair (bp) *EcoRI*-*PvuII* fragment from the cDNA p1.6 CR2 (33) into the *SmaI* site of the expression vector pGEM-3Z (Promega). PG3Z/CR2 was linearized with *HindIII*, and radioactive RNA probes were synthesized with T7 RNA polymerase as described by Melton et al. (20); 85 µCi of [α -³²P]dCTP (400 Ci/mmol) was used in a 25-µl reaction mix and supplemented with 10 µM unlabeled CTP.

Hybridization and RNA digestion. Hybridizations were performed by mixing 4.0×10^5 cpm of ³²P-labeled RNA probe with 30 µl of hybridization solution (80% formamide–0.4 M NaCl–1 mM EDTA–40 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid), pH 6.7]) containing total RNA. The solution was heated to 95°C for 8 min and immediately transferred to a water bath maintained at 45°C and incubated overnight. Three hundred microliters of 10 µM Tris (pH

7.5)–5 mM EDTA–200 mM NaCl–100 mM LiCl containing 20 µg of RNase A (type III A; Sigma) per ml and 200 IU of RNase T₁ (Boehringer) per ml was added, and the reaction mixes were incubated for 1 h at room temperature. The digestions were terminated by the addition of 10 µl of 20% SDS and 2.5 µl of proteinase K (Sigma) at 20 mg/ml and further incubated for 15 min at 37°C. After phenol-chloroform extraction and ethanol precipitation, the precipitated RNA was dissolved, denatured, and fractionated on a 5% polyacrylamide–8 M urea sequencing gel and electrophoresed at 30 V/cm. The gel was then exposed to X-ray film at –70°C.

DNA isolation and Southern blot. Total cellular DNA was extracted and purified by the classical phenol-chloroform extraction protocol (19). From 5 to 10 µg of DNA was digested with 50 IU of restriction endonuclease *BamHI* or *EcoRI* (Bethesda Research Laboratories) following conditions described by the manufacturer. Digested DNAs were either electrophoresed on a 1% (wt/vol) agarose gel in TBE buffer (0.089 M Tris borate, 0.089 M boric acid, 0.002 M EDTA) or directly applied to a GeneScreen Plus filter for dot blotting. After electrophoresis, DNA was hydrolyzed for 15 min in 0.25 N HCl and denatured for 30 min in TS (0.4 N NaOH, 6 M NaCl) before being transferred on GeneScreen Plus filter in TS. After transfer, the membrane was treated for 10 min in NS (0.5 M Tris hydrochloride [pH 7], 1 M NaCl) and dried at room temperature. Labeling of the probes and hybridization were performed as described above for RNA analysis. EBNA2A (U430; Cordier et al., submitted for publication) and EBNA2B (pJ-HKA7; described in reference 1) probes were kindly provided by G. Bornkamm.

Detection of soluble CD23 in conditioned medium from transplanted NPC cells. C15 nude mice tumors (1 cm³) were minced into 1-mm³ pieces, which were washed two times in RPMI 1640 medium (Imperial Laboratories). Viable cells were dispersed by pipetting tumor fragments in a solution containing 0.14 M NaCl, 6 mM KCl, 5.5 mM NaHCO₃, 6 mM EDTA, 5.5 mM glucose, and 2×10^{-6} M trypsin (Choay, France). Cells were transferred into 5 ml of fetal calf serum (FCS; Biological Industries) at 4°C to stop trypsin hydrolysis. After filtration through sterile wire gauze, cells were washed, centrifuged, and resuspended in RPMI medium. Approximately 5×10^7 viable cells were obtained from C15 tumors. The monocellular suspension was incubated in RPMI medium supplemented with 1 mM pyruvate, 20 mM glucose, 2 mM glutamine, 50 mg of gentamicin per liter, and 10% FCS at a concentration of 1×10^6 or 5×10^5 cells per ml in 24-well plates. Conditioned supernatants were cleared by filtration on a 0.22-µm membrane (Millipore). A 96-well plate (Intermed) was first incubated with 50 µl per well of 2 µg of purified anti-CD23 monoclonal antibody MA25 (kindly provided by J. Y. Bonnefoy [2]) per ml in 0.1 M carbonate buffer (pH 9.6) at 4°C overnight. The wells were then washed five times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (Tween-PBS); washing was always performed with this buffer and coated with PBS containing 2% bovine serum albumin (BSA) for at least 4 h at room temperature. After five washings, 100-µl samples were added to the wells and the plates were incubated at 4°C overnight. Plates were then washed five times, and 50 µl of alkaline phosphatase-coupled anti-CD23 monoclonal antibody H107 (kindly provided by J. Yodoi [21]), diluted at 2 µg/ml with 2% BSA-PBS, was added to each well. The plates were then incubated overnight at 4°C, washed five times, and supplemented with 100 µl of *p*-nitrophenyl phosphate (1 mg/ml) (Sigma) diluted with 9.7% diethanolamine, 3 mM

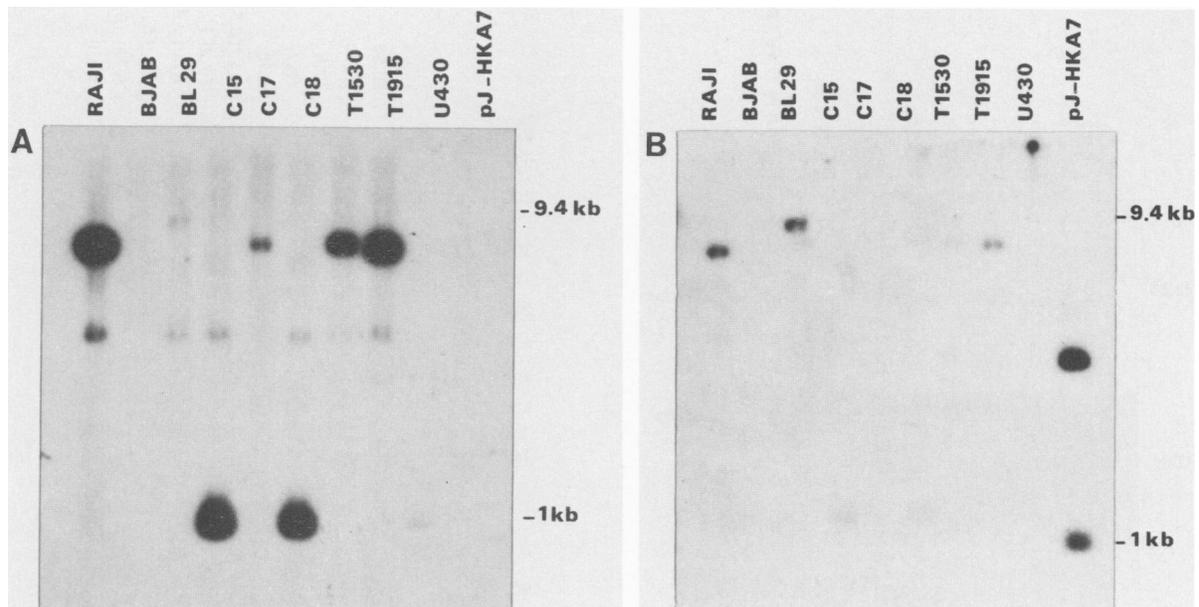


FIG. 1. Southern blot detection of EBV genomes. DNA (10 μ g) (*Bam*HI digested) from different cell lines was analyzed. Raji is an EBV-positive EBNA2 type A Burkitt's lymphoma line. BL29 is an EBV-positive EBNA2 type B Burkitt's line. BJAB is an EBV-negative Burkitt's line. C15, C17, C18, T1530, and T1915 are EBV-positive NPC tumors. U430 and pJ-HKA7 are two plasmids containing EBNA2A and EBNA2B coding sequences, respectively (1; Cordier et al., submitted). (A) A probe specific for the coding sequence of EBNA2A was hybridized. (B) The same filter was rehybridized with a probe specific for the EBNA2B coding sequence (see Materials and Methods).

NaN₃, and 0.5 mM MgCl₂ · 6H₂O at pH 9.8. After incubation at room temperature, the A₄₀₅ was determined with a Titertek Multiscan (Flow Laboratories).

Preparation of anti-EBNA2A and anti-EBNA1 rabbit sera and analysis by immunoblotting. (i) **Anti-EBNA2A rabbit serum.** The 4.71-kb *Sph*I DNA restriction fragment (positions 49650 to 54360 on the EBV genome physical map) from the EBV M-ABA genome was cloned into the tryptophan-regulated *Escherichia coli* expression vector pATH11 (T. J. Koerner, unpublished results). This construct encodes a fusion protein containing an amino-terminal part of the anthranilate synthase of *E. coli* and 140 amino acids of the COOH-terminal part of the EBNA2A protein. This fusion protein was used for rabbit immunization as described recently (23).

(ii) **Anti-EBNA1 rabbit serum.** The *Hind*III I1 fragment from the EBV M-ABA genome corresponding to the EBNA1 open reading frame was cloned in the pEX vector (27) and then used to transform *E. coli* NF1 bacteria. The fusion protein was induced as described before (23) and used to immunize rabbits.

Electrophoresis and immunoblotting. Cells were washed in PBS and solubilized at a concentration of 10⁷ cells per ml of sample buffer (10% sucrose, 0.06 M Tris [pH 7], 0.002 M EDTA, 2% SDS, 0.002 M phenylmethylsulfonyl fluoride, 2% 2-mercaptoethanol) and boiled for 10 min. Then, 8 μ l of a protein solution with an optical density at 280 nm of 0.8 was subjected to a discontinuous gel with a stacking gel of 5% (wt/vol) acrylamide and a resolving gel of 10% (wt/vol) acrylamide. After polyacrylamide gel electrophoresis separation, proteins were transferred electrophoretically to nitrocellulose overnight at 45 V. The blotted filters were incubated for 1 h with PBS containing 10% (wt/vol) skimmed milk and then incubated for 2 h at room temperature with anti-EBNA2A rabbit serum diluted 1:100 in PBS-milk or anti-EBNA1 rabbit serum diluted 1:100 in PBS-milk. After

incubation with primary antibody, the filters were washed in PBS-0.1% (vol/vol) Tween 20 and incubated for 2 h with anti-rabbit immunoglobulin horseradish peroxidase-linked F(ab')₂ fragment (Sigma) diluted 1:500 in PBS-milk. After washes in PBS-Tween, the filters were stained for 10 min by the substrate solution containing 3,3'-diaminobenzidine tetrahydrochloride and H₂O₂.

In order to increase the sensitivity of EBNA2A detection, the peroxidase-antiperoxidase (PAP) technique was used. After incubation with the primary antibody and washing in PBS-Tween, the filter was incubated for 1 h with swine anti-rabbit immunoglobulin serum (Dakopatts) diluted 1:100 in PBS-milk and then washed and incubated for 2 h with rabbit PAP (Dakopatts) diluted 1:400 in PBS-milk. The filter was washed and stained as described above.

RESULTS

Detection of EBV genomes and characterization of EBNA2 genes in transplanted NPC tumors. DNA extracted from the five transplanted NPC tumors and from three Burkitt's lymphoma cell lines (Raji, BL29, and BJAB) was digested with *Bam*HI and hybridized with two cloned probes from the EBNA2 gene: EBNA2 gene type A (U430 [Cordier et al., submitted]) and EBNA2 gene type B (pJ-HKA7 [1]). By comparison with Raji, which is a Burkitt's lymphoma line with 50 copies of the EBV genome per cell, we estimated that C15, C18, T1530, and T1915 contained between 15 and 35 EBV copies per cell, whereas C17 contained between 2 and 5 EBV copies (Fig. 1A). Transplanted NPC tumors hybridized only with the EBNA2A probe (Fig. 1A) and not with the EBNA2B probe (Fig. 1B). Furthermore, C15 and C18 hybridization revealed an additional *Bam*HI site in EBNA2 gene giving a signal of ca. 1 kb (Fig. 1A). This EBNA2 polymorphism has already been detected in the M-ABA EBV genome (1).

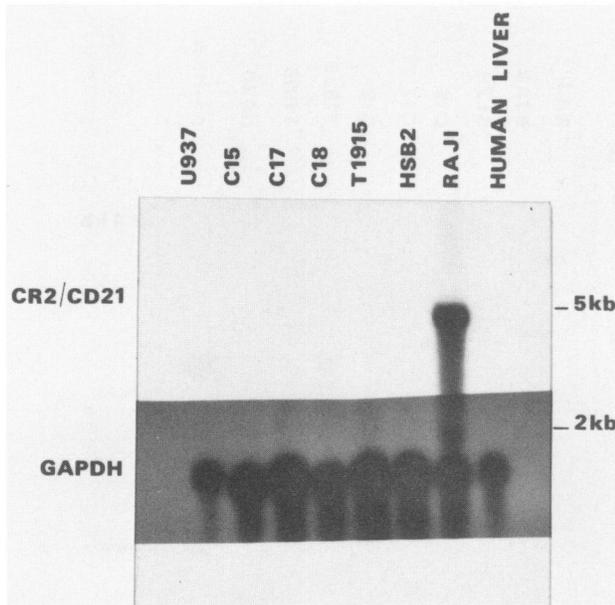


FIG. 2. RNA blot analysis of NPC tumors probed with CR2 cDNA and exposed for 3 days in autoradiography. A 30- μ g amount of each RNA was analyzed in each lane. The level of CR2 mRNA in each cell line was compared with the level of transcription of the housekeeping gene for GAPDH. U937 is a monocytic cell line, HSB2 is a T-cell line, and Raji is an EBV-positive Burkitt's lymphoma line. C15, C17, C18, and T1915 are EBV-positive NPC tumors passaged in nude mice.

CR2 transcription analysis in transplanted NPC tumors.

RNA extracted from the five NPC cell lines was probed with a 1.6-kb cDNA fragment subcloned from the CR2-coding clone λ 4.11 (33). No signal was detected by Northern blot analysis even after long exposure of the films. On the other hand, a specific 5-kb mRNA was expressed in Raji and not in HSB2 cells, a T-cell line not expressing CR2. A cDNA probe coding for the housekeeping protein GAPDH hybridized to a 1.4-kb mRNA in each lane of the filter (Fig. 2).

To improve the sensitivity of our transcriptional assay, RNase mapping experiments were performed since this technique allows us to detect as little as 0.1 pg of specific RNA (20). With an autoradiographic exposure of 3 days, the 450-bp CR2 band corresponding to the *EcoRI-PvuII* fragment subcloned from the cDNA p1.6 CR2 (33) was detected in BL36 and Raji cells, two EBV-positive Burkitt's lymphoma lines, and not in tRNA, a negative control (Fig. 3). Different quantities of nonselected Raji cell RNA were tested in amounts ranging between 20 and 0.5 μ g to estimate the threshold of CR2 detection. The specific CR2 fragment was readily detected with 1 μ g or more of Raji RNA but not with 0.5 μ g of RNA (Fig. 3). A faint CR2 transcript was detectable at this level in C15 and C18 but not in C17. The intensity of the signal obtained with NPC RNA was weaker than that obtained with 1 μ g of Raji RNA but similar to those obtained with BL70, an EBV-negative Burkitt's lymphoma line (Fig. 3).

Blast2/CD23 transcription analysis. Northern blots were hybridized with the pFc ϵ R1 insert, a full-length cDNA encoding the human low-affinity Fc ϵ receptor (18). Three NPC cell lines (C15, C17, and T1915) were found to transcribe the 1.7-kb mRNA (Fig. 4). This transcript was also detected in Raji cells, a positive control, and not in HSB-2

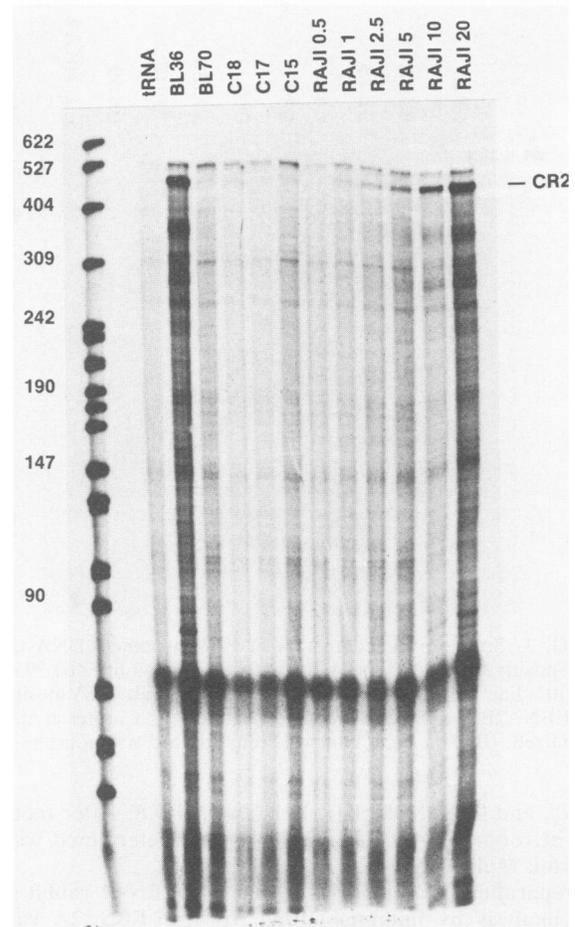


FIG. 3. RNase protection analysis of EBV receptor CR2 mRNA. Total RNA was annealed to the RNA probe, which contained the sequence corresponding to the 450-bp *EcoRI-PvuII* fragment of the p1.6 CR2 cDNA (33), and then digested with RNases A and T₁. The sizes of protected bands were analyzed by polyacrylamide gel electrophoresis and exposed 3 days in autoradiography. A 30- μ g amount of total RNA was analyzed in each lane, and different RNA quantities prepared from Raji cells were tested in a range between 20 and 0.5 μ g (20, 10, 5, 2.5, 1, and 0.5 μ g). All different dilutions of Raji RNA were completed up to 30 μ g with tRNA. tRNA is a negative control. BL36 and Raji are two Burkitt's lymphoma lines associated with EBV and CR2 positive. BL70 is an EBV-negative Burkitt's lymphoma line. C15, C17, and C18 are EBV-associated NPC lines. Molecular size markers are pBRR322 cut by *MspI* (sizes in base pairs).

cells, a known Blast2/CD23-negative cell line. No major variations of transcription were detected during the passages of the NPC cell lines in nude mice. However, we found variations of Blast2/CD23 mRNA expression in C17 correlated with the duration of tumor growth in nude mice (data not shown). RNA extracted from different nonhematopoietic malignant cell lines, including sarcoma, carcinoma, and neurological tumors, did not hybridize with the pFc ϵ R1 probe (Fig. 5), whereas total RNA and polyadenylated [poly(A)⁺] RNA from C15 did. Blast2/CD23 mRNA cannot be expressed from contaminant murine tissues, since RNA extracted from nude mice tissue failed to hybridize with the pFc ϵ R1 probe.

Detection of soluble form of Blast2/CD23 protein in trans-

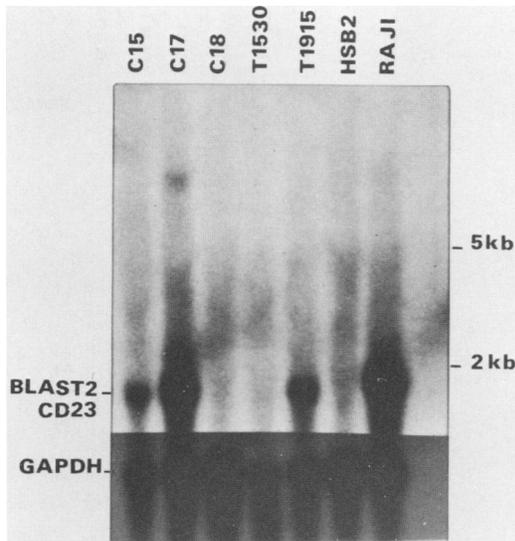


FIG. 4. RNA blot analysis of NPC tumors probed with pF_ceR1 cDNA (CD23) and exposed for 3 days in autoradiography. A 30- μ g amount of each RNA was analyzed in each lane. The level of CD23 RNA in each cell line was compared with the GAPDH mRNA level. HSB2 is a T-cell line, and Raji is an EBV-positive Burkitt's lymphoma line. C15, C17, C18, T1530, and T1915 are EBV-positive NPC tumors passaged in nude mice.

planted NPC tumors. Blast2/CD23 antigen was not detected by immunofluorescence with three distinct monoclonal antibodies either on collagenase- and trypsin-treated cells or on acetone-fixed C15, C17, and C18 cells (data not shown).

To assess whether Blast2/CD23 protein was present in the soluble form in spite of the negative results obtained by immunofluorescence staining of cells, we developed an enzyme-linked immunoassay. With this sandwich immunoassay technique, using two anti-CD23 monoclonal antibodies, MAb25 (2) and H107 (21), we were able to clearly identify soluble Blast2/CD23 (SCD23) in C15 culture supernatant (Fig. 6). SCD23 was present in large quantities in the supernatant of BL2 cells converted with the B95/8 EBV strain, whereas no SCD23 was detected in HT29 cell (colonic carcinoma) supernatant conditioned and prepared under the same conditions (Fig. 6). SCD23 was also detected in C17 supernatant, with quantitative variations comparable to those observed in the transcription study, and was undetectable in C18 supernatant (data not shown).

Analysis of EBNA2A and EBNA1 protein expression in transplanted NPC tumors. DNA Southern analysis revealed that the five NPC cell lines harbored an EBV with an EBNA2 gene of type A. We therefore investigated by immunoblotting to see whether our NPC cell lines expressed the EBNA2A protein and whether such expression was correlated with CD23 expression. The rabbit sera specific for the EBNA2A protein detected a molecule of ca. 80 kDa in the EBV-associated lymphoid lines JI, Raji, and BL60 (Fig. 7A). As expected, EBNA2A was not detected in the EBV-negative Burkitt's lymphoma line BL41 nor in CEM, a malignant T-cell line. EBNA2A was not detected in any of the five transplanted NPC tumors tested (Fig. 7A). To estimate the integrity and the quantity of the proteins loaded, the filters hybridized with an EBNA1 rabbit antiserum clearly detected the 70-kDa EBNA1 protein in the five NPC and all EBV-positive cell lines tested (Fig. 7B).

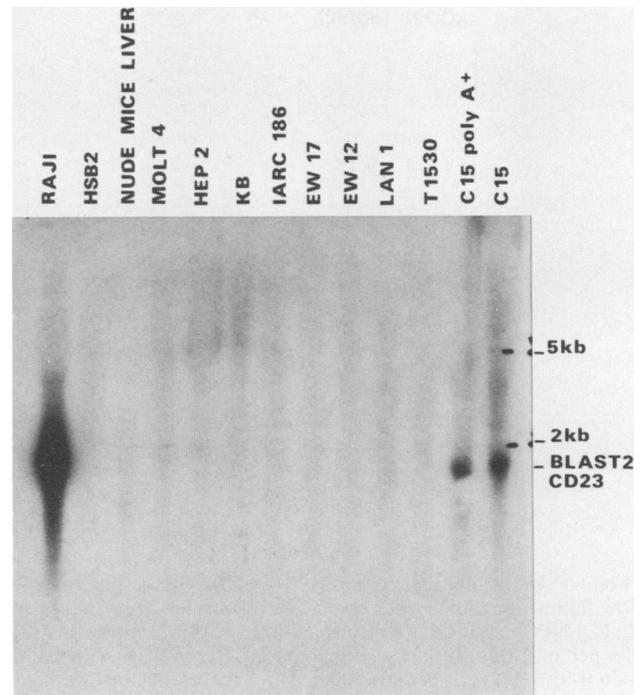


FIG. 5. RNA blot analysis of various cell lines probed with pF_ceR1 cDNA (CD23). A 40- μ g amount of each RNA was analyzed in each lane. Raji is an EBV-positive Burkitt's lymphoma line; HSB2 and MOLT4 are T-cell lines; HEP and KB are carcinoma cell lines; IARC 186 is a Wilms' tumor line; EW17 and EW12 are Ewing's sarcoma cell lines; LAN1 is a neuroblastoma cell line; T1530 and C15 are NPC tumors passaged in nude mice. A 5- μ g amount of poly(A)⁺ RNA from C15 was analyzed.

DISCUSSION

Our experiments establish that the EBV receptor CR2 is transcribed at very low levels in certain EBV-positive NPC lines. CR2 mRNA was not detectable by the usual Northern analysis, whereas by using the more sensitive RNase mapping assay (20), two of the three NPC lines tested were found to transcribe the CR2 gene. The sizes of the protected bands corresponding to CR2 mRNA were found to be identical in EBV-associated lymphoid and in NPC lines. The intensity of the signal obtained with C15 and C18 was lower than that obtained with 1 μ g of nonselected RNA prepared from the CR2-EBV-positive lymphoma line Raji and was equivalent to those of BL70, an EBV-negative Burkitt's lymphoma line. Transcriptional quantification showed that steady-state CR2 mRNA levels were approximately 50 times lower in C15 and C18 than in Raji. Therefore, it appears that levels of CR2 mRNA found in malignant EBV-associated NPC epithelial cells differ from those in EBV-positive B-cell lines, where CR2 mRNA is readily detectable (Calender et al., submitted for publication; M. Billaud, unpublished data).

It has been reported that frozen sections of basal or suprabasal layers of normal pharyngeal epithelium express a molecule which reacts with HB-5, B2, and AB-1, three anti-CR2 monoclonal antibodies (34, 36). However, immunoprecipitation of labeled epithelial cells with MAb anti-CR2 yields a protein of 200 kDa which seems to be antigenically related but not identical to the 145-kDa EBV receptor CR2 (36). Cervical epithelial cells possess an EBV receptor, since they are capable of infection by and replicate EBV (24, 26).

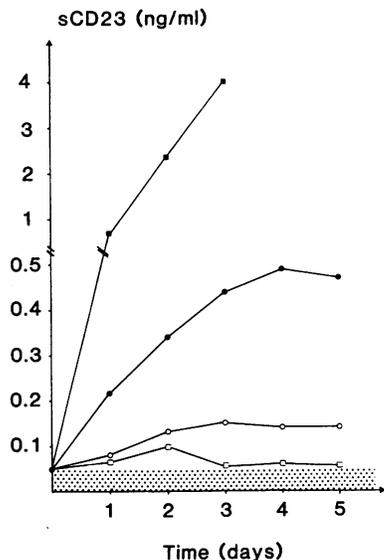


FIG. 6. Detection by immunoassay of SCD23 in the supernatant of the following cell lines: (□) colonic carcinoma HT29 (10^6 cells per ml); (○) NPC tumor C15 (10^5 cells per ml); (●) NPC tumor C15 (10^6 cells per ml); (■) Burkitt's lymphoma BL2 converted with EBV strain B-95/8 (10^6 cells per ml). Shaded area represents the threshold of SCD23 detection.

This raises the possibility that the uterine cervix is a site for virus shedding (25). However, clear-cut results have not been obtained for CR2 expression on NPC cells (13, 14). It is noteworthy that single-cell suspensions as well as tumor imprints of C15, C17, and C18 were found to be CR2 negative when tested with OKB7 and HB-5, two monoclonal antibodies which recognize distinct epitopes of the CR2 molecule (4). This discrepancy between the RNA and protein data is reminiscent of EBV conversion of EBV-negative lymphoma cells. EBV-negative lymphoma cell lines such as BL70, which we tested in this study, express very low levels of CR2 molecules but are nevertheless infectable by EBV (6). Since CR2 transcription was found to be similar in C15 and C18 to that in BL70, we assume that, as in the conversion model mentioned above, subdetectable levels of CR2 proteins are expressed by NPC cells. Further experiments will establish whether such low CR2 expression could permit EBV infection of malignant epithelial nasopharyngeal cells.

Upon EBV infection *in vitro*, human B lymphocytes are transformed to a state of continuous proliferation through the activation of a B-cell proliferation pathway, involving the up expression of the molecule Blast2/CD23 (29, 30). Blast2/CD23 is a leukocyte differentiation antigen which is the same molecule as the low-affinity receptor for the Fc portion of human immunoglobulin E (2, 37). Blast2/CD23 is postulated to play an important role in EBV-induced B-cell immortalization, since its shed form possesses B-cell growth factor activity (28), which could be involved in the autocrine loop activated by the virus (15). Although Blast2/CD23 was not detected on the surface of NPC cells by the usual immunofluorescence staining techniques, we cannot exclude the possibility that Blast2/CD23 was released directly into the supernatant of short-term cultures of NPC tumors. We now report that these malignant epithelial cells express the 1.7-kb Blast2 mRNA and that the soluble form of Blast2/CD23 protein is detectable in NPC tumor supernatants. Expression was not found in the other nonhematopoietic cancers tested.

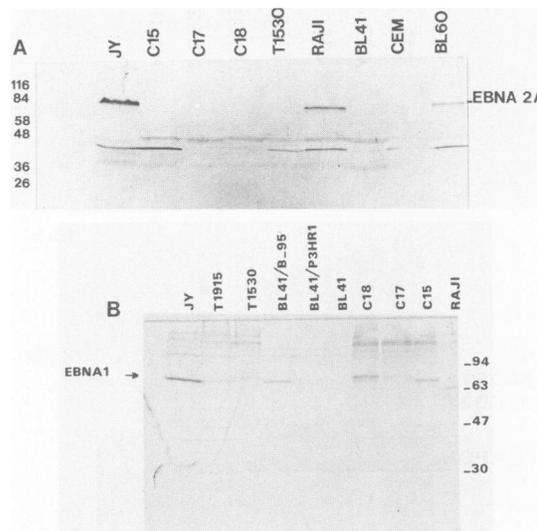


FIG. 7. Immunoblot analysis of EBNA2A (A) and EBNA1 (B) proteins. Immunoblots were revealed with polyclonal rabbit sera anti-EBNA2A and anti-EBNA1 described in Materials and Methods. In order to increase the sensitivity of our assay, PAP was used to reveal the EBNA2A immunoblot. (A) JY is an EBV-positive lymphoblastoid cell line. C15, C17, C18, and T1530 are EBV-positive NPC tumors passaged in nude mice. RAJI and BL60 are EBV-positive Burkitt's lymphoma lines. BL41 and CEM are an EBV-negative Burkitt's lymphoma line and a malignant T-cell line, respectively. (B) C15, C17, C18, T1530, and T1915 are EBV-positive NPC tumors passaged in nude mice. BL41/P3HR1 and BL41/95 are the BL41 cell line converted with P3HR1 and B-95 strains of EBV, respectively. Sizes are in kilodaltons.

Blast2/CD23 expression was also not detected in CNE-1 and CNE-2 (Rousselet et al., submitted for publication), two malignant epithelial cell lines established from differentiated NPC biopsies (38) which lack the EBV genome, suggesting that EBV infection may be critical in Blast2/CD23 induction. EBNA2 is a candidate in this transactivation process, since its expression turns on Blast2/CD23 expression in lymphoid cells (6, 31; Cordier et al., submitted). In contrast to the lymphoid system, the expression of Blast2/CD23 in NPC cells was not correlated with the expression of this viral protein. This suggests that if Blast2/CD23 expression depends on EBV, it might be mediated through EBV proteins other than EBNA2. Indeed, the pattern of EBV gene expression in NPC cells was reported to be different from that of lymphoid cells with down expression of EBNA2, -3, and -5 but frequent expression of latent membrane protein (LMP) and constant expression of EBNA1 (10, 35). Recently, the introduction of LMP by gene transfer into a human EBV-negative Burkitt's lymphoma line was demonstrated to increase the expression of Blast2/CD23 (32). The 62-kDa LMP protein was clearly identified in C15, weakly identified in C17, and barely detectable in C18 and T1530, but the LMP status of T1915 has not been resolved (P. Busson and M. Billaud, manuscript in preparation). Therefore, Blast2/CD23 activation in NPC cells could be mediated through LMP expression. However, it remains to be explained why C18 and T1530, which seem to express low levels of LMP, do not express Blast2/CD23. Alternatively, one can postulate that the NPC progenitor cells have an epithelial differentiation pattern which involves Blast2/CD23 expression. Regarding this hypothesis, one should note that Blast2/CD23, postu-

lated to be a specific leukocyte marker, has recently been shown to be expressed at high levels on follicular dendritic cells (17). Consequently, with regard to the difficulty of detecting Blast2/CD23 by immunofluorescence on NPC cell membranes despite observed expression, either an alternative splicing removes the transmembrane coding exon of CD23 mRNA or destabilization of the membrane form of Blast2/CD23 protein involves rapid cleavage and shedding of the soluble moiety. Finally, we and others have demonstrated that in NPC cells, as in the lymphoid system, interleukin-4 induces Blast2/CD23 expression, and induction is blocked by interferon gamma (Rousselet et al., submitted).

Since C18 and T1530 do not express Blast2/CD23 protein, different NPC cell lines exist. In addition, Blast2/CD23 has been reported to be spatially associated with human leukocyte antigen (HLA) DR on cell surfaces (3), and C15 and C17 consistently coexpress both HLA DR and Blast2/CD23, whereas C18 does not (4). C15 and C17 also express CDW40 antigen and soluble factors with interleukin-1 activity, whereas C18 cells do not express these molecules (4). Transplanted NPC tumors also provide a means of investigating whether soluble Blast2/CD23 is able to act synergistically with other growth factors, such as molecules with interleukin-1 activity which may be present in the supernatant of NPC cultures (5), to promote growth of these epithelial cells.

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