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Expression of Epstein–Barr virus lytic gene BRLF1 in nasopharyngeal carcinoma: potential use in diagnosis

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Tumour cells of undifferentiated nasopharyngeal carcinoma (NPC) consistently harbour Epstein–Barr virus (EBV) genes. Expression of mRNA transcripts associated with EBV latency has been demonstrated in such cells. However, expression of EBV lytic genes has not been well elucidated, although various lines of evidence have suggested that there is EBV replication in NPC tumour cells. We have studied mRNA expression of representative EBV lytic genes by RT–PCR in nasopharynx biopsies obtained from NPC and control individuals. In both NPC and control biopsies, EBV lytic genes BZLF1, BALF2 and BCLF1 were detected readily. However, BRLF1 was detected in NPC biopsies only. The BRLF1 gene was then cloned and expressed *in vitro*, and the protein product, Rta, was used as an antigen to detect specific antibodies by immunoprecipitation in plasma samples obtained from NPC patients and healthy controls. IgG antibodies directed against Rta were detected in 44 of 53 NPC plasma samples (83·0%), but only in 1 of 53 control samples (1·9%). Furthermore, the antibody binding regions were found in the C-terminal two-thirds of Rta. This serological result confirms indirectly that BRLF1 is specifically expressed in NPC tumour cells. Rta might play an important role in NPC pathogenesis, considering its multiple functions in EBV replication and cell cycles. Moreover, the detection of IgG antibodies directed against Rta could be developed into a diagnostic parameter for NPC.

Introduction

Epstein–Barr virus (EBV) is a ubiquitous gammaherpesvirus that infects more than 90% of the human population. The primary infection is generally asymptomatic or causes infectious mononucleosis in some circumstances (Rickinson & Kieff, 1996). A lifelong viral persistence is established as a form of *in vivo* latency in healthy carriers following primary infection, with low copies of episomal virus maintained in resting memory B cells (Babcock *et al.*, 1998; Tierney *et al.*, 1994; Anagnostopoulos *et al.*, 1995). Spontaneous reactivation of the latent infection can occur in oropharyngeal epithelium (Sixbey *et al.*, 1984) and/or local mucosal lymphoid tissues (Babcock *et al.*, 1998; Anagnostopoulos *et al.*, 1995), possibly by responding to physiological signals such as cytokines, steroid hormones and CD40 ligand (Thorley-Lawson *et al.*, 1996). Disruption of latency in EBV-transformed lymphoblastoid cells *in vitro* can be induced by treatment with various chemicals, including phorbol esters, n-butyrate, calcium ionophores and anti-immunoglobulin (Rickinson & Kieff, 1996; Thorley-Lawson *et al.*, 1996). EBV utilizes a variety of distinct genetic programmes, each of which is categorized by expression of a unique set of viral genes, at various stages of its life-cycle. These programmes can be grouped into either the latency-associated programme or the replicative programme (Rodriguez *et al.*, 1999).

EBV is associated with a spectrum of malignancies of lymphoid and epithelial cell origin (Rickinson & Kieff, 1996), such as Burkitt's lymphoma, T-cell lymphoma, Hodgkin's disease, undifferentiated nasopharyngeal carcinoma (NPC) and gastric carcinoma. NPC is the most consistently EBV-associated malignancy and occurs mainly among the Chinese population in Southern China and Southeast Asia. However, the EBV genome has been detected in malignant epithelial cells in NPC patients regardless of geographical origin (Wolf *et al.*, 1973). The demonstration of monoclonality of the viral DNA indicates that the malignancy has arisen from clonal expansion of a single EBV-infected progenitor cell (Raab-Traub & Flynn, 1986). EBV latency-associated gene expression is consistently

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detected in NPC tumour cells and is regarded therefore as one of the factors in the oncogenesis (Brooks *et al*., 1992). However, various lines of evidence indicate that reactivation of EBV can also occur in NPC tumour cells. Infectious EBV can be isolated from NPC tumour cells (Trumper *et al*., 1977), and the linear form of the EBV genome, which is indicative of the productive cycle, can be detected in NPC tumour tissues in spite of the presence of the predominant episomal form (Raab-Traub & Flynn, 1986). Levels of serum immunoglobulin A (IgA) directed against EBV lytic gene products, the early antigen (EA) and the viral capsid antigen (VCA), are elevated in NPC patients, and have been used as serological markers for diagnosis (Henle & Henle, 1976). Moreover, cell-free EBV DNA has been detected in the plasma of most NPC patients, indicating productive virus release into the blood (Lo *et al*., 1999).

Upon EBV reactivation, two key immediate early (IE) lytic genes, BZLF1 and BRLF1, encoding Zta (BZLF1 transcription activator) and Rta (BRLF1 transcription activator) respectively, are transcribed, and consequently activate several downstream viral promoters and lead to an ordered cascade of viral gene expression (Kieff, 1996). Recently, Rta was reported to disrupt viral latency in an epithelial cell-specific fashion (Zalani *et al*., 1996). Another study further proposed that Rta is sufficient for disruption of latency in both B lymphocytes and epithelial cells, although much higher levels of Rta were needed for efficient lytic cycle induction in B lymphocytes (Ragoczy *et al*., 1998). These *in vitro* experiments suggest that there might be a difference in EBV replication mode between B cells and epithelial cells. As previously reported, EBV replication in healthy people occurs mainly in B cells residing in the oropharynx (Babcock *et al*., 1998; Anagnostopoulos *et al*., 1995; Thorley-Lawson *et al*., 1996). NPC tumour tissues could be representative of EBV lytic replication in epithelial cells. By using biopsy tissues obtained from these two groups of people, it might be possible to identify whether there is indeed a difference in EBV replication between B and epithelial cells. We performed such a study, and found expression of EBV lytic genes, BZLF1, BALF2 and BCLF1, in both the NPC and the control biopsies. However, BRLF1 could be detected in NPC biopsies only, suggesting that *in vivo* EBV replication in NPC tumour epithelial cells differs from that in EBV-harbouring non-malignant cells in the control individuals. This finding is very interesting and meaningful, since the specific expression of EBV BRLF1 mRNA and especially its protein product, Rta, could be regarded as a tumour antigen for NPC, and therefore have potential use in NPC diagnosis.

Methods

■ **Samples and cell line.** All NPC blood samples and nasopharyngeal biopsies were collected from the ENT clinics of the Singapore National University Hospital and the Singapore General Hospital. Blood samples were taken from 53 histologically confirmed NPC patients prior to radiotherapy and 53 healthy EBV-seropositive volunteers. Plasma was collected after centrifugation at 1500 r.p.m. for 5 min and stored at −20 °C until use. Mononuclear cells (MNC) were isolated from anti-coagulant blood of 10 NPC patients and 19 healthy volunteers by density gradient centrifugation over Ficoll–Histopaque (Sigma) and stored in liquid nitrogen until use. Nasopharyngeal biopsies were collected from 7 untreated patients histopathologically confirmed as undifferentiated NPC and 5 individuals with non-specific reactive inflammation of the pharynx as controls, and were snap-frozen and stored in liquid nitrogen until use. For the 5 control individuals, IgA response to EBV EA and VCA was examined by an indirect fluorescent method (Feng *et al*., 1999). Anti-VCA was positive in all five cases (1:10 to 1:640 dilution), but none was positive for anti-EA.

B95-8 is an EBV-positive marmoset B cell line with approximately 5% of the cells in replication cycle. Cells were maintained in RPMI 1640 growth medium (GibcoBRL) supplemented with 10% FCS, 2 mM glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Extensive EBV replication was induced by treating the cells with 20 ng/ml 12-O-tetradicanoylphorbol 13-acetate (TPA) and 3 mM sodium butyrate when needed.

■ **RT–PCR and hybridization.** Total RNA was isolated from snap-frozen biopsies and aliquots of 5×10^6 MNC with a high RNA isolation kit (Boehringer Mannheim), according to the manufacturer's instructions and a procedure described previously (Hu *et al*., 1999). To avoid DNA contamination, all RNA samples were treated with RNase-free DNase at 37 °C for 30 min. cDNA was synthesized in a 20 μl reaction mixture containing 2 μg of RNA as template using oligo-p$(dT)_{15}$ primer and AMV reverse transcriptase (Boehringer Mannheim). To detect expression of BZLF1, BRLF1, BALF2 and BCLF1 mRNA, primers for two rounds of PCR and an internal oligonucleotide probe for Southern blotting were synthesized according to previously published sequences (Prang *et al*., 1997). Procedures for RT–PCR and hybridization have been described previously (Hu *et al*., 1999). Briefly, 1 μl of the cDNA was added to a final volume of 50 μl PCR reaction mixture containing dNTP (0·2 mM), first round sense and antisense primers (0·5 μM each) and 1 unit of DynaZyme II DNA polymerase (Finnzymes). After being heated to 94 °C for 5 min, amplification was carried out for 35 cycles each consisting of 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 1 min. Except for the internal control of the house-keeping gene histone 3.3, a nested PCR was performed in the presence of the second round primers and 1 μl of the first-round PCR product as templates, with an additional 20 cycles of amplification under the same conditions as described above. RNA isolated from B95-8 cells was used as a positive control for RT–PCR detection of EBV lytic gene expression. Negative controls were performed in the absence of cDNA templates.

PCR products were separated on 1·7% agarose gel. After denaturation and neutralization, DNA was transferred from the gel onto a nylon membrane, and pre-hybridized in a buffer containing 5× Denhardt's solution, 6× SSPE, 0·1% BSA and 0·02% SDS at 42 °C overnight. The internal oligonucleotide probe end-labelled with digoxigenin 11-dUTP (DIG) (Boehringer Mannheim) was added and allowed to hybridize at 42 °C for 1 h. The blot was washed with 2× SSPE containing 0·1% SDS at the melting temperature of each probe for 10 min and washed twice with 2× SPSS at room temperature. After blocking with buffer A (0·1 M maleic acid, 0·15 M NaCl; pH 7·5) supplemented with 1% blocking reagents (Boehringer Mannheim) at room temperature for 1 h, the membrane was incubated with alkaline phosphatase-conjugated anti-DIG monoclonal antibody at room temperature for 30 min, followed by washing twice with buffer A containing 0·3% Tween 20 and equilibrating with a buffer containing 0·1 M Tris–HCl (pH 9·5) and 0·1 M NaCl for 3 min. Finally, chemiluminescent alkaline phosphatase substrate CSPD (Boehringer Mannheim) was added, and the signal was detected by exposing the membrane to X-ray film.

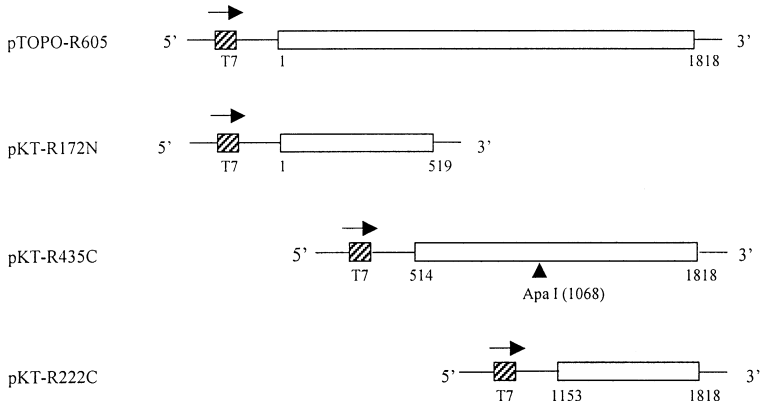

Fig. 1. Construction of plasmids encoding the full-length and truncated forms of the BRLF1 gene. The full-length BRLF1 gene was amplified by RT–PCR, and cloned into pTOPO vector. This cloned gene was used as template for PCR to generate various fragments of BRLF1, and these fragments were subsequently cloned into pKT vector for *in vitro* transcription and translation.

■ **Plasmid construction.** Plasmid pTOPO-R605 (Fig. 1), containing the 1818 bp full-length BRLF1 cDNA, was constructed by PCR using template cDNA synthesized from B95-8 mRNA of and the TOPO TA Cloning Kit (Invitrogen) according to the manufacturer's instructions. The primers used were 5′ CCGGAATTCATGAGGCCTAAAAAGG-ATGGCTT 3′ (sense), and 5′ TGCTCTAGACTAAAATAAGCTGG-TGTCAAAAATAG 3′ (antisense). Vector pKT for subcloning was derived from plasmid pING14 (Liu *et al.*, 1994). pKT-R172N, pKT-R435C and pKT-R222C constructs contained three truncated BRLF1 fragments under the control of T7 promoter (Fig. 1). pKT-R172N contained the 519 bp N-terminal fragment of BRLF1, which was amplified by PCR using pTOPO-R605 plasmid DNA as template with primers 5′ TAATACGACTCACTATAGGG 3′ (sense) and 5′ CTGTTGGATCCT-TACACTACCTGCTTGCC 3′ (antisense). The fragment was digested with *Eco*RV and *Bam*HI and cloned into *Pvu*II/*Bam*HI-digested pKT vector. pKT-R435C was cloned using pTOPO-R605 plasmid DNA as template with primers 5′ GCCTTCCATGGCAGCGGTCCACCAA 3′ (sense) and 5′ TGCTCTAGACTAAAATAAGCTGGTGTCAAAAAT-AG 3′ (antisense), which amplified a DNA fragment of 1305 bp. pKT–R222C was cloned with primers 5′ GCCTTCCATGGCAGCGG-TCCACCAA 3′ (sense) and 5′ TGCTCTAGACTAAAATAAGCTGG-TGTCAAAAATAG 3′ (antisense), which amplified a DNA fragment of 666 bp. In these two constructs, *Nco*I and *Xba*I restriction enzyme sites were incorporated at the end of the primers, and used for cloning.

■ ***In vitro* translation.** Plasmid DNA was isolated from *E. coli* TOP10F′ or JM101 by using the Qiagen plasmid Midi Kit, and adjusted to a final concentration of 1 μg/μl. Plasmid DNA was transcribed and translated *in vitro* in the presence of [^{35}S]methionine, using the TnT system (Promega) according to the manufacturer's instructions. Each 50 μl of TnT lysate reaction contained 25 μl rabbit reticulocyte lysate, 1 μl T7 polymerase, 1 μl amino acid mixture minus methionine (1 mM), 4 μl [^{35}S]methionine (10 mCi/ml), 2 μl RNasin ribonuclease inhibitor and 2 μg linearized plasmid DNA. Furthermore, plasmid pKT-R435C was partially digested at the unique *Apa*I site to obtain an additional internal R185I fragment (Fig. 1). The TnT reaction was performed at room temperature for 1·5 h and stopped by adding an equal volume of 100 μg/ml RNase A and 10 mM EDTA (pH 7·5–8·0).

■ **Immunoprecipitation.** TnT product (5 μl) was added to 95 μl of a dilution buffer (0·14 M NaCl, 10 mM Tris–HCl, pH 8·0, and 0·5% Nonidet P-40). After vortexing, 5 μl of plasma was added and incubated at room temperature for 30 min; 80 μl of protein A–Sepharose beads (100 mg/ml; Sigma) was then added and incubated with rotation at room temperature for 30 min. The beads were pelleted and washed three times with RIPA buffer (50 mM Tris–HCl, pH 8·0, 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate and 1% SDS), and then resuspended in 35 μl of loading buffer and boiled for 5 min. After centrifugation, supernatants were resolved on SDS–PAGE (12·5% polyacrylamide). The gel was fixed in a solution containing 10% acetic acid and 50% methanol for 30 min, amplified with Amplify solution (Amersham) for 30 min, dried and finally exposed to X-ray film.

■ **Statistical analysis.** Differences in anti-Rta antibody reactivity between NPC and healthy individuals were compared by Chi-square test. $P < 0{\cdot}05$ was considered to be significant.

Results

Expression of EBV lytic genes in peripheral blood

To detect expression of EBV lytic gene mRNA, we established a sensitive two-round RT–PCR method followed by hybridization with an internal oligonucleotide probe according to a previous report (Prang *et al.*, 1997). As shown in Fig. 2(*a*) and Table 1. Expression of EBV IE gene BZLF1 was detected in 4 of the 10 (40%) NPC patients, and 10 of the 19 (53%) healthy carriers. Expression of early gene BALF2 was detected in 3 of the 10 NPC patients, and 1 of the 19 controls. However, expression of the other EBV IE gene, BRLF1, was not detected in the samples from either NPC or control individuals. Expression of EBV late gene BCLF1, which is an indicator of complete lytic cycle, was not observed in any sample. These results reveal that complete EBV replication does not occur in the blood lymphocyte population in either NPC or healthy individuals, although there may be abortive replication.

EBV reactivation occurs in the nasopharynx of both NPC and control individuals, but expression of BRLF1 could be detected only in NPC biopsies

EBV lytic gene expression was further examined in nasopharynx biopsies obtained from 7 NPC patients and 5 controls. The results are shown in Fig. 2(*b*) and Table 1. BZLF1, BALF2 and BCLF1 mRNA expression was similar in both the

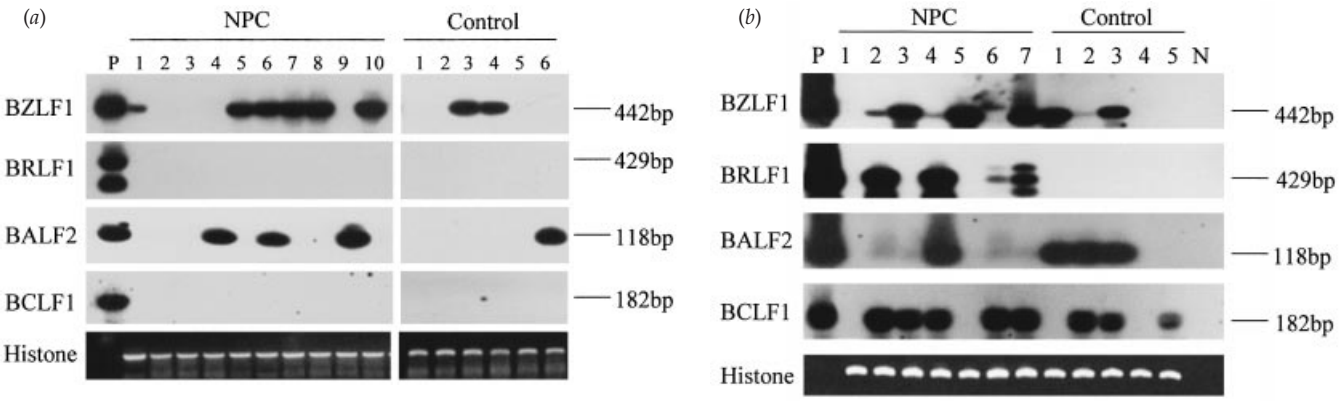

Fig. 2. Expression of EBV BZLF1, BRLF1, BALF2 and BCLF1 mRNA genes in blood and biopsies obtained from NPC and control individuals. (*a*) Representative results from the blood samples. (*b*) Biopsy results. Numbers indicate individual samples. Total RNA was isolated from the biopsies and treated with DNase I before cDNA synthesis. The above-mentioned genes were amplified with previously described primer sets for 36 cycles, and 1 μl of the product was used for a second round amplification with nested primers for 20 cycles. The PCR products were separated on agarose gel, and transferred to nylon membrane for hybridization with an internal oligonucleotide probe. A PCR for histone was used to indicate that the RNA samples were intact and the input was similar for each reaction. RNA isolated from cell line B95-8 was used in the positive control (P), and in the negative control no cDNA was added.

Table 1. Expression of EBV lytic genes in blood lymphocytes and nasopharyngeal biopsies

	Peripheral blood lymphocytes		Biopsies	
	NPC ($n = 10$)	Control ($n = 19$)	NPC ($n = 7$)	Control ($n = 5$)
BZLF1	4	10	5	3
BRLF1	0	0	4	0
BALF2	3	1	5	3
BCLF1	0	0	5	3

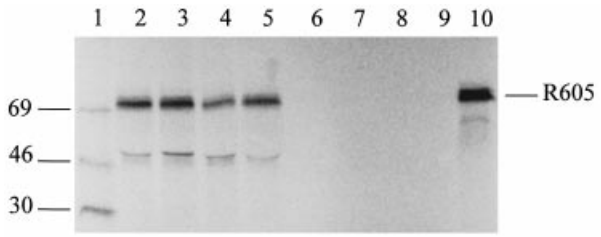

Fig. 3. Representative results of immunoprecipitation to detect anti-Rta IgG (IgG-Rta) in plasma samples obtained from NPC and control individuals. Full-length Rta (R605) was produced with the *in vitro* TnT system in the presence of [^{35}S]methionine (lane 10). Immunoprecipitation was performed in the presence of 5 μl of radiolabelled R605, 5 μl of plasma and 80 μg of protein A–Sepharose beads. Precipitated proteins were resolved on SDS–PAGE and the gel was exposed to X-ray film. Lanes 2–5 show samples obtained from NPC patients, and lanes 6–9 samples from controls. Lane 1, molecular mass markers (kDa).

NPC patients and the controls. BZLF1 expression was detected in 5 of the 7 NPC biopsies and 3 of the 5 control biopsies. BALF2 was expressed in 5 NPC and 3 control biopsies, and expression of the late gene BCLF1 was observed in 5 NPC and 3 control biopsies. In sharp contrast, expression of the IE gene BRLF1 was detected in 4 of the 7 NPC tumour tissues, but in none of the 5 control biopsies. For detection of BALF2 and BCLF1, an additional experiment was performed to exclude the possibility of DNA contamination, because there was no intron located within the amplified region between the two primers. RNA samples isolated from NPC biopsy 4 and control biopsy 2 (Fig. 2), which were positive for both BALF2 and BCLF1, were pretreated with 5 μg/ml RNase at 37 °C for 30 min, followed by cDNA synthesis and RT–PCR. As expected, no positive signal for both BALF2 and BCLF1 could be detected (data not shown). These results reveal that complete EBV replication can occur in the nasopharynx of both NPC and control individuals, although the replication mode might be different, and BRLF1 seems to be involved only in EBV replication in NPC tumour tissues.

Detection of antibodies directed against BRLF1 gene product Rta

The entire BRLF1 open reading frame was cloned into pCRII-TOPO vector under the control of the T7 promoter. Transcription and translation of this construct in an *in vitro* TnT system in the presence of [^{35}S]methionine resulted in the detection of a product with a molecular mass of approximately 70 kDa (Fig. 3, lane 10). This should represent the full-length Rta. The radiolabelled Rta was used in an immunoprecipitation assay to detect anti-Rta IgG (IgG-Rta) antibodies. Plasma samples from 53 NPC patients prior to therapy and 53 healthy controls were used. Representative results from 4 NPC patients and 4 controls are shown in Fig. 3. IgG-Rta was detected in

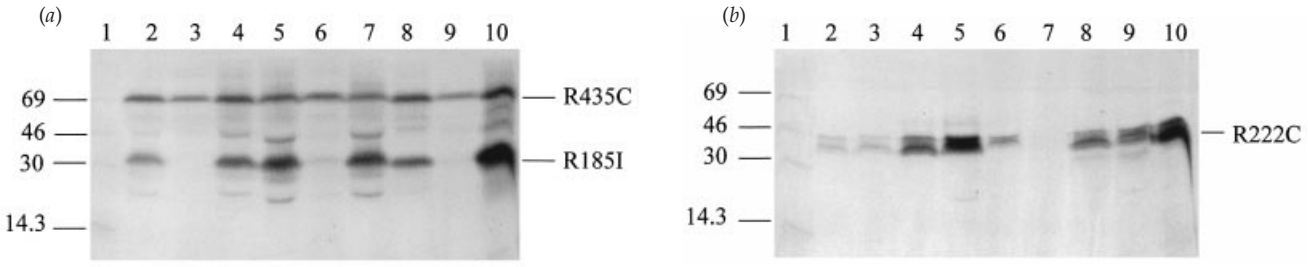

Fig. 4. Representative results of immunoprecipitation to determine the antibody-binding regions in Rta. (*a*) An N-terminal truncated BRLF1 gene, pKT-R435C, encoding 435 amino acids was constructed as depicted in Fig. 1. After partial digestion with *Apa*I, the plasmid DNA was used for transcription and translation with the *in vitro* TNT system in the presence of [^{35}S]methionine, which generated two proteins, R435C and R185I (lane 10). Plasma samples from NPC patients were used for the immunoprecipitation assay. Lanes 2–9 show that these plasma samples contained antibodies that precipitated R435, and samples in lanes 2, 4, 5, 6, 7, and 8 contained antibodies that precipitated R185I. (*b*) An N-terminal truncated construct, pKT-R222C, was made, and *in vitro* translated into a protein, R222C, that contained the C-terminal 222 amino acids of Rta (lane 10). This protein was used to detect antibodies directed at this region of Rta. The plasma samples used as in (*a*). Except for lane 7, plasma samples all precipitated R222C.

plasma from 44 of the 53 NPC patients (83%), but in only 1 of the 53 healthy individuals (1·9%). This difference was statistically significant ($P < 0{\cdot}01$).

Defining the antibody-binding regions

We next defined the regions responsible for inducing this specific IgG-Rta. Three truncated BRLF1 fragments were subcloned into pKT vector. pKT-R172N was employed to produce a C-terminal truncated protein R172N. pKT-R435C and pKT-R222C constructs were used to generate two N-terminal truncated proteins, R435C and R222C (Figs 1 and 4). After partial digestion of pKT-R435C at the *Apa*I site, an internal fragment of Rta (R185I) was obtained together with R435C as a mixture (Figs 1 and 4). R172N, R222C and the mixture of R435C and R185I were used to define the antibody-binding regions by immunoprecipitation. Twenty-five IgG-Rta positive plasma samples were randomly selected for the experiment. Representative results are depicted in Fig. 4. All 25 plasma samples efficiently precipitated R435C (Fig. 4*a*). Of these 25 samples, 20 samples precipitated the internal R185I (Fig. 4*a*). Ten samples were used to precipitate the C-terminal R222C (Fig. 4*a*), and 9 showed a positive result. However, none of the 10 samples used to precipitate N-terminal R172N yielded any positive signals (data not shown). These results indicate that the antibody-binding regions reside in the two-thirds C-terminal part of Rta, and that at least two antibody-binding regions exist in this fragment. Approximately 70% of the positive plasma samples contain antibodies recognizing both regions, while about 30% recognize only one of the two regions.

Discussion

By detecting mRNA expression from four EBV lytic genes, BZLF1, BRLF1, BALF2 and BCLF1, which are indicative of immediate early (BZLF1 and BRLF1), early and late stages of the lytic cycle (Rickinson & Kieff, 1996), respectively, we found a different picture of virus replication in peripheral blood and nasopharyngeal biopsies. Expression of IE gene BZLF1 and early gene BALF2 was detected occasionally, while signals for late gene BCLF1, an indicator of complete lytic cycle, was not detectable in blood. Therefore, EBV remains in the latent state in blood of both NPC patients and healthy individuals, although abortive lytic replication could occur sporadically. These results are consistent with the currently accepted concept that in healthy carriers EBV persists in a latent state in resting B cells (Babcock *et al.*, 1998; Tierney *et al.*, 1994; Anagnostopoulos *et al.*, 1995; Sixbey *et al.*, 1984). Lo *et al.* (1999) recently reported that by using a real-time PCR method cell-free EBV DNA was detectable in the blood of 96% of NPC patients, but in only 7% of the controls, indicating there is a very active EBV replication in NPC patients. However, as our study shows that EBV replication does not occur in the blood of NPC patients, this active replication must occur in other tissues. Among these the tumour tissue is the most possible site. Our RT–PCR results confirmed this assumption. The EBV lytic genes BZLF1, BRLF1, BALF2 and BCLF1 were all frequently detected in the biopsy tissues, showing that complete EBV replication occurs here, and this is probably the main source of the cell-free EBV DNA in the blood of NPC patients. Martelrenoir *et al.* (1995) previously reported similar results in studying EBV lytic replication in 8 NPC biopsies. They found BZLF1 expression in all 8 biopsies, and BRLF1 in 3 of the 8 biopsies (1995). However, considering that the biopsy tissue could contain resident B lymphocytes that may harbour EBV, we specifically included non-NPC biopsies as controls in the present study. In these control biopsies, expression of BZLF1, BALF2 and BCLF1 was detected, confirming that EBV replication occurs in the oropharynx. However, in healthy individuals this replication is at a much lower rate, since only in a small proportion of the healthy carriers could EBV DNA be detected in the blood (Lo *et al.*, 1999). Interestingly, comparing the expression pattern of lytic genes, an obvious difference is the expression of BRLF1 in

NPC but not in the controls, suggesting a different mode of virus reactivation in NPC tumours. Specific BRLF1 expression at the protein level was further confirmed indirectly by the detection of IgG antibodies directed against Rta in plasma from most NPC patients (83%). This antibody response coincides with mRNA expression, since in contrast to the high positive rates in the NPC patients, only 1 serum sample was positive among the 53 healthy controls. This also correlates with the previous studies, which showed that expression of BRLF1 seemed to be more efficient in disrupting latent infection in epithelial cells than in B lymphocytes (Zalani *et al.*, 1996; Ragoczy *et al.*, 1998). Therefore, in NPC patients, viral reactivation occurs in local tumour tissues with a specific expression of BRLF1, which subsequently results in induction of a strong humoral immune response to its protein product.

Expression of BRLF1 may play an important role in the regulation of viral reactivation and in the development of NPC. BZLF1 and BRLF1 are expressed simultaneously within 2 h of induction of EBV replication (Takada & Ono, 1989). Rta can act alone or synergistically with Zta to induce maximal activation of several viral promoters that are essential for EBV replication, including BMLF1, BMRF1, BHRF1 and the EBV DNA polymerase gene (Kenney *et al.*, 1989; Holley-Guthrie *et al.*, 1990; Cox *et al.*, 1990; Liu *et al.*, 1996). Rta also has a profound effect on cell cycle regulation (Henderson *et al.*, 1993; Gutsch *et al.*, 1994; Swenson *et al.*, 1999; Zacny *et al.*, 1998), and this might contribute to the oncogenesis of NPC. The gene product of BHRF1, one of the Rta-responsive lytic genes, is known as a viral homologue of proto-oncogene *bcl*-2, and can protect cells from apoptosis (Henderson *et al.*, 1993). Rta can activate the c-*myc* gene (Gutsch *et al.*, 1994) and induce elevation of E2F1 (Swenson *et al.*, 1999), a cellular factor important for proliferation. Together with its ability to inactivate the retinoblastoma protein (Rb), a crucial cell cycle suppressor, Rta can efficiently activate S-phase entry during viral lytic infection (Swenson *et al.*, 1999; Zacny *et al.*, 1998).

The expression of BRLF1 in NPC patients may have clinical uses, for instance as a diagnostic parameter for NPC. Our preliminary data have shown that the IgG antibodies directed against Rta can serve as a serological parameter for NPC diagnosis and screening in a susceptible population. This parameter seems to be highly specific, and the sensitivity could be further improved by using a larger amount of serum, which in this study was 5 μl diluted into 100 μl of the reaction buffer. However, it is not practical to use the present immunoprecipitation protocol for routine testing in a serological laboratory. Since we have identified the antibody-binding regions in Rta, it should be possible to develop a simpler method such as ELISA for detecting the IgG antibodies in the blood. We have previously reported that there are a large number of activated infiltrating T cells in the NPC tumour tissues, and many of these cells secrete interferon-γ (Tang *et al.*, 1999). It will be interesting to investigate whether there are Rta-specific T cells among these infiltrating cells. In fact, multiple epitopes of Rta can be recognized by *in vitro* generated EBV-specific cytotoxic T cells from blood of healthy EBV-seropositive adults (Pepperl *et al.*, 1998).

In conclusion, we have demonstrated the expression of EBV lytic gene BRLF1 in NPC patients. The expression of its product, Rta, may facilitate tumour cell growth and consequently contribute to the disease progress. The detection of IgG antibodies directed against Rta in NPC patients can be developed into a diagnostic parameter.

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