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Epstein–Barr Virus Gene Expression in Nasopharyngeal Carcinoma

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SUMMARY

Epstein–Barr virus (EBV), an agent with growth transforming potential for human B cells, is associated with certain B cell lymphomas in man and also with an epithelial tumour, undifferentiated nasopharyngeal carcinoma (NPC). Since B cell growth transformation is associated with the constitutive expression of a small number of EBV-coded latent proteins, the nuclear antigens EBNA 1, EBNA 2, EBNA 3 and EBNA-LP and the latent membrane protein (LMP), the present work sought to determine whether this same pattern of virus gene expression occurred in NPC. Tumour biopsies were taken from NPC patients from three areas of differing tumour incidence (Kenya, Algeria, Britain) and immediately snap-frozen, as were biopsies of non-EBV-related carcinomas for controls. Immunoblotting of PAGE-separated proteins with selected human sera identified 24 NPC biopsies clearly expressing EBNA 1. When the analysis was extended using selected human sera with antibodies against the other EBNAAs, there was no detectable expression of EBNA 2, EBNA 3 or EBNA-LP in any of these 24 biopsies; their EBNA 2-negative status was confirmed using a monoclonal antibody (MAb) PE2 which was reactive in immunoblotting and in immunoprecipitation with EBNA 2A and EBNA 2B proteins. Similar experiments with two different LMP-specific MAbs, CS1 to 4 and S12, revealed heterogeneity between NPC biopsies; 9/24 biopsies were demonstrably LMP-positive, the degree of expression varying considerably between individual tumours in a manner which was not related to the level of EBNA 1 expression. None of the 24 NPC biopsies expressed detectable amounts of EBV lytic cycle antigens. A nude mouse-passaged NPC cell line, C15, likewise expressed EBNA 1 and LMP but none of the other EBV latent proteins nor lytic cycle antigens. This work identifies a novel type of EBV–cell interaction in NPC cells which is distinct from that seen in in vitro transformed B cell lines and from that seen to date in EBV-positive B cell lymphomas.

INTRODUCTION

The Epstein–Barr virus (EBV), a human herpesvirus with potent cell growth transforming ability, is a B lymphotropic agent best known for its association with endemic Burkitt’s lymphoma (Epstein & Achong, 1979) and with the B cell lymphomas to which immunosuppressed patients are particularly prone (Cleary et al., 1986). Despite this predilection for the B cell system, the strongest and best documented link between EBV and a human cancer involves an epithelial tumour, undifferentiated nasopharyngeal carcinoma (NPC). The association with NPC, first revealed by serological evidence (Old et al., 1966), was later reinforced by nucleic acid hybridization data indicating that EBV DNA was consistently detectable in NPC biopsy material (zur Hausen et al., 1970; Nonoyama et al., 1973). Separation of the malignant epithelial cells from infiltrating lymphocytes either by cell culture (Wolf et al., 1973; Huang et al., 1974) or
by passage in nude mice (Klein et al., 1974) subsequently showed that the EBV genome, and indeed an EBV-coded nuclear antigen (EBNA), could be detected in the tumour cells.

Since that time the association between EBV and the undifferentiated forms of NPC (WHO histological subtypes 2 and 3) has been confirmed for many different racial groups, whether these exhibit a high, intermediate or low incidence of the tumour; all cases examined were consistently positive for the EBV genome in the tumour and a characteristic serological picture with IgA antibodies to virus lytic cycle antigens (Henle & Henle, 1976; de Thé, 1982; Zeng, 1985). Certain other carcinomas of tonsillar (Brichacek et al., 1984), supraglottic laryngeal (Brichacek et al., 1983), parotid (Saemundsen et al., 1982) and thymic (Leyvraz et al., 1985) epithelium also appear to show an association with this virus, but in these cases the evidence is less extensive and the consistency of the association has yet to be established.

Very little is known about the precise role of EBV in the pathogenesis of NPC, indeed the whole study of EBV–epithelial cell interactions is still in its infancy. Only recently has there been convincing evidence to show that the virus is capable of replicating in oral/pharyngeal epithelium in vivo (Sixbey et al., 1984; Greenspan et al., 1985). Furthermore it has been quite difficult to reproduce this cycle of events by infecting normal epithelial cells in vitro (Sixbey et al., 1983, 1987), probably because the progress of the infectious cycle is critically dependent upon the ordered differentiation of stratified epithelium. At the moment there is no in vitro system for the EBV-induced growth transformation of human epithelium, but the evidence from the well-characterized B cell system would imply that a restricted set of viral proteins has cell growth transforming potential, i.e. the ‘latent’ gene products recognized by their constitutive expression in EBV-transformed lymphoblastoid cell lines (reviewed by Dambaugh et al., 1986). The EBV latent proteins include the nuclear antigens EBNA 1 encoded by the BKRF1 reading frame of the viral genome (Summers et al., 1982; Baer et al., 1984), EBNA 2 encoded by BYRF1 (Dambaugh et al., 1984), EBNA 3 encoded by a BLRF3/BERF1 spliced sequence (Hennessy et al., 1986; Kalin et al., 1986) and EBNA-LP encoded by highly spliced exons from the BamHI WY region of the genome (Dillner et al., 1986; Wang et al., 1987) as well as a latent membrane protein (LMP) encoded by BNLF1 (Hennessy et al., 1984). There is a certain amount of structural diversity between the EBV latent gene products encoded by different EBV isolates, but the only known example of major antigenic variation involves the EBNA 2 protein which exists either as an 85K (EBNA 2A) or as a 75K (EBNA 2B) species depending upon the isolate (Dambaugh et al., 1984).

Initial studies on RNA preparations from NPC biopsies have suggested that EBV gene expression in these tumour cells may follow a pattern similar to that seen in EBV-transformed lymphoblastoid cell lines, although some tumours also showed transcription from regions of the genome the expression of which is normally associated with entry into the lytic cycle (Raab-Traub et al., 1983; Tugwood et al., 1987). In the absence of a detailed study of the transcripts, however, these groups were unable to specify the precise coding sequences being expressed. There has been no corresponding study of EBV protein expression in NPC; indeed the detection of ‘EBNA’ staining by the anti-complement immunofluorescence test (Klein et al., 1974; Huang et al., 1974) constitutes the only available evidence for any EBV antigen being expressed in fresh NPC material, and it is still not clear whether one or more of the EBNAs contributed to this staining. In the present work we specifically address the question of EBV latent and lytic cycle antigen expression in NPC biopsies, using as probes selected human antisera and monoclonal antibodies with defined reactivities against EBV gene products.

**METHODS**

*Preparation of tumour tissues.* NPC biopsy specimens were obtained from patients from the Meru district of Kenya attending the Kenyatta National Hospital, Nairobi, from Algerian patients being treated at the Institut Gustav Roussy, Paris, and from a Caucasian patient attending the Queen Elizabeth Hospital, Birmingham. These represent patients drawn from high, intermediate and low risk groups respectively (Clifford, 1972; Muir, 1972; Waterhouse et al., 1982). All NPC specimens entering this study were classified as the undifferentiated form of the tumour (WHO 3), the histopathology of the tissue being evaluated at source. The EBV-positive C15 tumour line, derived from an undifferentiated NPC in an Algerian patient, was established in nude mice in the Institut Gustav Roussy, Paris (Busson et al., 1988) and was subsequently passaged in nude mice in the Department of Cancer
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Studies, Birmingham. Specimens of rectal (ReCa), colonic (CoCa, AdCa) and cervical (CxCa) carcinomas were used as control tissues in this study, and a nude mouse-passaged colonic carcinoma was used as a control for the C15 tumour.

Biopsy specimens were halved and either prepared for histology or immediately snap-frozen and maintained at −70 °C. After shipment to Birmingham, frozen specimens were thawed, weighed and dissected into 2 mm3 cubes. The tissue was then suspended in RIPA buffer (0.1% SDS, 0.5% deoxycholate, 0.5% Triton X-100, 0.4 M NaCl, 5 mM EDTA, 20 mM-Tris-HCl pH 7.6) to give a 10% (w/v) solution. This suspension was then mixed using an Ultra-turrax homogenizer (Janke & Kunkel). After sonication the samples were divided and (i) stored at −20 °C for immunoprecipitation, (ii) diluted 1:1 with gel sample buffer (2 × GSB; 0.02% bromophenol blue, 4% SDS, 4% 2-mercaptoethanol, 20% glycerol, 50 mM-Tris-HCl pH 6.8), boiled and stored at −20 °C for immunoblotting (iii) incubated with Protein A-Sepharose CL-4B (Bioprocessing) for 1 h at 4 °C then treated as in (ii) above.

B cell lines. EBV-positive control lines used in these experiments included X50-7 and JC-5, in vitro transformed latently infected lymphoblastoid cell lines carrying EBNA 2A-encoding and EBNA 2B-encoding virus isolates respectively (Rowe et al., 1987) and the virus producer cell line P3HR1 which carries an EBNA 2 deletion mutant (Bornkamm et al., 1982). EBV-negative control lines included IARC-BL2 and -BL30 (Lenoir et al., 1985).

Antisera and monoclonal antibodies (MAbs). Expression of nuclear antigens EBNA 1, EBNA 2, EBNA 3 and EBNA-LP in the various tumour specimens was determined by immunoblotting with selected sera (Mo, PK, RM, JL, Am) having strong reactivity against one or more EBNA, often with relatively weak reactivity against lytic cycle antigens. Expression of these antigens was independently checked by immunoblotting with a human serum EE the predominant reactivity of which was against the early antigen (EA) and virus capsid antigen (VCA) complexes of the virus productive cycle. Each of these sera have already been well characterized in the context of earlier work (Rowe et al., 1987a; Rowe et al., 1987; Gregory et al., 1987).

MAbs were also used to probe for EBNA 2 for, and for EA(D). Expression of EBNA 2 was detected using the MAbs PE2 which recognizes both the EBNA 2A and EBNA 2B proteins (L.S. Young et al., unpublished data). Expression of LMP was detected using either a pool of four MAbs (CS1 to 4) (Rowe et al., 1987b) or the S12 MAb (Mann et al., 1985). Expression of EA(D) was detected using the MAbs R3 (Pearson et al., 1983). MAb screening for EBNA 2 and LMP was by immunoblotting and by immunoprecipitation, and screening for EA(D) was by immunoblotting.

Immunoblotting. Protein samples from tumour specimens were separated by discontinuous PAGE and blotted onto nitrocellulose as previously described (Rickinson et al., 1987). Excess protein-binding sites on blotted filters were blocked by incubation for 2 h in 20 mM-Tris-buffered saline pH 7.5, containing 5% dried skimmed milk (TBS-milk). The filters were subsequently incubated with antibody for 16 h at 4 °C. For this purpose the human sera were used at 1:100 dilution, the MAbs PE2 and CS1 to 4 were used at 1:10 and 1:15 dilutions of culture supernatant, and the MAbs S12 and R3 were used at 1:500 and 1:200 dilutions of ascitic fluid, all dilutions being made in TBS-milk. After incubation with the primary antibody the filters were washed in phosphate-buffered saline with 0.1% Tween 20 (PBS–TWEEN). Where MAbs were used as the primary antibody, the filters were subsequently incubated for 1 h at room temperature with rabbit anti-mouse immunoglobulin (Dakopatts) and washed again in PBS–TWEEN; a second-step antibody was not used with human sera. In all cases specifically bound antibody was then detected by incubating filters for 2 h with 125I-labelled Protein A (Amersham) diluted to 0.1 μCi/ml in TBS-milk. Following extensive washing in PBS–TWEEN the filters were dried and subjected to autoradiography for 1 to 5 days with an intensifying screen. Molecular weight determinations were made from protein standards (Sigma) which had been prestained with remazol dye and run on the same gel.

Immunoprecipitation. Solubilized tumour specimens (100 to 200 μl) were added to 500 μl of diluent (1% NP40, 3% bovine serum albumin, 0.4 M NaCl, 20 mM-Tris–HCl pH 7.2) together with 10 μl of normal rabbit serum. The extracts were incubated for 45 min at 4 °C. Protein A-Sepharose CL-4B was then added and after 1 h at 4 °C the supernatants were removed and incubated overnight with the appropriate MAbs at 4 °C by end-over-end rotation. Protein A-Sepharose was coated with rabbit anti-mouse immunoglobulins (Dakopatts) and added to the extracts. After a 2 h incubation at 4 °C the Sepharose beads were washed three times in wash buffer (1% NP40, 5% sucrose, 0.4 M NaCl, 5 mM-EDTA, 20 mM-Tris–HCl pH 7.2) and subsequently heated at 100 °C for 2 min in 1 × GSB. Samples were separated and immunoblotted as described and the filters incubated with the appropriate primary antibody.

Antibody elution. Elution of antibodies from immunoblots was performed as described by Rhodes et al. (1987). Nitrocellulose filters blotted with PAGE-separated extracts from either control cell lines or NPC specimens were incubated overnight with Mo serum. A 1 cm wide strip was then cut from one end of the filter, washed and subsequently incubated with a 1:1000 dilution of alkaline phosphatase-conjugated anti-human IgG (Sigma) for 2 h at room temperature and the remaining portion of the filter was returned to Mo serum. Bands on the 1 cm wide strip were detected using 5 mM-Tris–HCl pH 8.8, 2 mM-MgCl2, 0.2 mg/ml z-naphthyl phosphate and 0.5 mg/ml Fast Red Salt (Sigma). The larger portion of the filter was then removed from the serum, washed in PBS–TWEEN and aligned with the smaller developed strip. Horizontal pieces corresponding to the visualized bands were cut in the larger filter. These were then incubated with 1 ml of 50 mM-diethylamine, 150 mM NaCl pH 11.5 for 30 min at
room temperature. The solution was then neutralized with 0.2 M-phosphate buffer pH 2.0 and bovine serum albumin (Sigma) was then added to a final concentration of 200 μg/ml. The solution was then diluted 1:4 in TBS-milk and used to reprobe another blotted nitrocellulose filter holding appropriate PAGE-separated extracts.

RESULTS

Immunoblotting for EBNA 1 with selected human sera

The initial survey of EBNA 1 expression was conducted on extracts of snap-frozen tissue from 29 cases of NPC; in each case histological examination of an adjacent piece of tissue from the same original biopsy sample had confirmed the presence of undifferentiated (WHO 3 histological subtype) NPC cells. Parallel extracts of various non-EBV-related human carcinomas were always included as controls in the immunoblotting experiments. The initial studies used a reference human serum (Mo) selected for its strong anti-EBNA 1 reactivity, its non-reactivity against other EBV latent proteins and its unusually low reactivity against antigens of the virus productive cycle (see Rowe et al., 1987a).

The immunoblotting results in Fig. 1 (a) show that serum Mo detected EBNA 1 in the reference EBV-transformed B cell line X50-7 and in the nude mouse-passaged NPC line C15. On the other hand it had no significant reactivity with extracts from four non-EBV-related carcinomas, three snap-frozen as flesh biopsies (RecCa, CxCa1, CxCa2) and one snap-frozen as nude mouse-passaged material (CoCa nude). EBNA 1 bands could be observed in several of the NPC biopsies examined in the same immunoblot (C157 to C182) but there was the occasional tumour (C 159) where no relevant signal could be found. Note that in Fig. 1 (a) the reactivity at 50K, well below the EBNA 1 size range, is non-specific and was observed both with fresh NPC and with fresh control carcinomas but not with any nude mouse-passaged material. This is almost certainly due to heavy chains of immunoglobulin molecules which contaminate fresh biopsy material and which react with the Protein A step of the immunoblotting procedure; thus the 50K band was markedly reduced if the biopsy extracts were pre-treated with protein A before loading onto the gel (data not shown).

Fig. 1 (b) presents the EBNA 1 immunoblotting results from a further series of 10 NPC biopsies (N 1 to N 10), showing firstly that the size of the major EBNA 1 band varied between individual biopsies and secondly that more than one band was often observed. Repeated testing showed that each individual biopsy gave the same characteristic pattern of bands when probed using Mo serum. Moreover other sera with reactivity against EBNA 1 but not against other EBV latent proteins also revealed the same combinations of bands for each biopsy, though the relative intensities of the bands sometimes differed (data not shown).

Sufficient biopsy material was available from one particular tumour (N5, lane 7) to allow the relationship between its two ‘EBNA 1’ bands, identified by Mo serum in Fig. 1 (b), to be studied further. The two bands were cut separately out of the gel, the antibodies binding to these bands were eluted and then the two eluates were used to reprobe gels containing extracts of the N5 tumour itself, of the reference EBV-transformed B cell line X50-7 and of appropriate EBV-negative controls (cell line BL2 and biopsy CxCa). Fig. 2 presents the results of this experiment, showing that both preparations of eluted antibodies were EBNA 1-specific since both recognized the two bands in the N5 biopsy extract as well as the characteristic EBNA 1 band in X50-7 cells.

Immunoblotting for EBNA 2, EBNA 3 and EBNA-LP with selected human sera

Twenty-four of the above 29 NPC biopsies contained detectable amounts of EBNA 1 protein and so these 24 (plus appropriate controls) were subsequently analysed for the expression of the other known virus-coded nuclear antigens EBNA 2 (A or B), EBNA 3 and EBNA-LP. Here the immunoblots were developed using human sera selected for their strong reactivity against one or more of these additional EBNA:s; the particular sera were RM (anti-EBNA 1, 2A, 3 and LP), PK (anti-EBNA 1 and 2A), JL (anti-EBNA 1 and 3) and Am (anti-EBNA 2B).

None of the defined EBNA proteins, except EBNA 1, could be detected in NPC biopsies by this method. Fig. 3 shows representative results obtained using serum RM on a range of NPC biopsies and on positive and negative controls. This serum detected EBNA 1, EBNA 2, EBNA 3
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Fig. 1. Expression of EBNA 1 in NPC biopsies. EBNA 1 was detected by SDS–PAGE and immunoblotting of tumour extracts with a human serum (Mo) having high anti-EBNA 1 reactivity, no reactivity against other EBNA species, and unusually low reactivity against antigens of the virus productive cycle. (a) EBNA 1 expression in the reference transformed B cell line X50-7 (lane 1), in nude mouse-passaged tumours (CoCa nude, lane 2; C15 nude, lane 3), in control carcinomas (RecCa, lane 4; CxcA 1, lane 5; CxcA 2, lane 6) and in NPC biopsies from Algerian patients (C157, C159, C161, C175, C180, C182; lanes 7 to 12). (b) EBNA 1 expression in NPC biopsies from Kenyan patients (N1 to N10; lanes 3 to 12). Lane 1 represents X50-7 and lane 2 the control carcinoma RecCa. Note that the reactivity at 50K is non-specific, being derived from the heavy chains of immunoglobulin molecules which contaminate fresh biopsy material and which cross-react in the Protein A step of the immunoblotting procedure.

and the characteristic ladder of EBNA-LP species in the reference EBV-transformed B cell line X50-7, and showed no significant reactivity with extracts of the non-EBV-related carcinomas. In the NPC biopsies, however, and in the nude mouse-passaged NPC line C15, the only specific bands detectable were those already identified as being EBNA 1-related (compare Fig. 3 with Fig. 1). The other human sera described above (except the anti-EBNA 1-negative Am serum) all detected EBNA 1 bands in the NPC biopsies but none of the other EBNA species were ever observed.
Fig. 2. Antibody elution experiment demonstrating EBNA 1 antigenicity of both upper (a) and lower (b) protein bands recognized by serum Mo in NPC biopsy extract N5 in Fig. 1(b) lane 7. The antibodies binding to the upper and lower bands were separately eluted and then used to reprobe filters containing SDS-PAGE-separated samples of NPC N5 (lanes 4), of the EBV-positive B cell line X50-7 (lanes 1) and of appropriate EBV-negative controls (BL2, lanes 2 and CxCa, lanes 3).

Fig. 3. Levels of EBNA 1, EBNA 2A, EBNA 3 and EBNA-LP expression in nude mouse-passaged tumours (CoCa nude, lane 2; C15 nude, lane 3), in a control tumour biopsy (AdCa, lane 4) and in NPC biopsies (C157, C175, C180, C182, lanes 5 to 8 and N2, N5, N6, N7, lanes 9 to 12) as detected by SDS-PAGE and immunoblotting. The blot was probed with a human serum (RM) having strong reactivity against EBNA 1, EBNA 2A, EBNA 3 and EBNA-LP. The 50K band represents cross-reactive immunoglobulin heavy chains. Lane 1 represents the reference cell line X50-7.

Immunoblotting/immunoprecipitation for EBNA 2 with MAb PE2

The availability of an anti-EBNA 2 MAb, PE2, which recognizes an epitope shared by the EBNA 2A and 2B proteins, allowed the question of EBNA 2 expression in NPC biopsies to be examined more extensively.

Fig. 4(a) serves to illustrate the efficiency with which MAb PE2 binds in immunoblotting to the EBNA 2 proteins encoded by prototype A (X50-7) and by prototype B (JC5) virus isolates, and confirms its lack of reactivity with the EBV-producer cell line P3HR1 carrying an EBNA 2 deletion mutant of the virus. When this MAb was used to probe the NPC biopsy extracts, there was no evidence of any detectable EBNA 2 protein in any of the tumours analysed. Fig. 4(b)
Fig. 4. (a) Immunoblotting reaction of anti-EBNA 2 MAb PE2 with extracts of B cell lines expressing either EBNA 2A (X50-7, lane 1) or EBNA 2B (JC5, lane 2) but not with extracts of EBNA 2-negative lines (P3HR1, lane 3; BL2, lane 4). (b) Immunoblotting reaction of PE2 with extracts of NPC biopsies (N2 to N10, B2; lanes 7 to 15, lane 16). Lane 5 represents the reference cell line X50-7 and lane 6 the carcinoma control RecCa. The very strong 50K reactivity from immunoglobulin heavy chains indicates the extent to which this gel has been over-exposed. (c) Immunoblots of NPC biopsy extracts N7 (lane 17) and N8 (lane 18) probed with the anti-LMP MAbs CS1 to 4, and similarly over-exposed.
shows a representative gel with 10 NPC biopsies (N2 to N10, B2) and appropriate positive and negative controls. The very strong 50K reactivity from immunoglobulin heavy chains is an indication of the extent to which this gel was over-exposed in an effort to detect an EBNA 2 signal. Nevertheless all the NPC lanes remained negative for EBNA 2, with the possible exception of a faint band at about 95K in one tumour, N7. Because this result with N7 was unique among the 24 NPC biopsies analysed, we carried out a second MAb immunoblotting experiment on selected NPCs including N7; the lanes were stained either with MAb PE2 or with the anti-LMP MAbs CS1 to 4. After long exposure, not only was the faint 95K band again observed with the N7 biopsy extract using PE2 (data not shown) but also an identical band was detected on the anti-LMP immunoblot well above the region of specific LMP reactivity (see Fig. 4c). The N7 biopsy result in Fig. 4(b) was therefore deemed to be non-specific rather than reflecting a positive signal for EBNA 2.

Immunoprecipitation studies with MAb PE2 on NPC biopsies likewise did not reveal any detectable expression of the antigen. As with the fresh NPC biopsies, the nude mouse-passaged NPC line C15 also gave no detectable expression of EBNA 2 by immunoblotting or by immunoprecipitation (data not shown).

Immunoblotting/immunoprecipitation for LMP with MAbs CS1 to 4 and S12

The 24 NPC biopsies were probed for LMP expression by immunoblotting with the pooled MAbs CS1 to 4 and with the independently derived MAb S12. Both reagents were raised against a bacterial fusion protein containing the carboxy half of the B95.8 strain LMP molecule and both recognize the LMPs encoded by virtually all EBV strains thus far tested (Mann et al., 1985; Rowe et al., 1987b).

There were marked differences between individual biopsies in terms of LMP expression; see for instance Fig. 4(c) where biopsy N8 is clearly expressing the protein but no specific signal is detectable from biopsy N7. Fig. 5 shows representative results from a wider panel of NPC biopsies (N2 to N16) immunoblotted with MAbs CS1 to 4 (Fig. 5a) and with MAb S12 (Fig. 5b). The two reagents produced essentially similar patterns of results, with S12 tending to be the more sensitive in most but not all cases. Characteristic LMP bands in the 60K to 70K region of the gel were detectable for biopsies N2, N14, N15 and N16; over-exposure revealed a faint LMP band in this same region with biopsy N5 whereas biopsies N7 and N11 remained negative (see also Fig. 4c). Note that where LMP expression was strong, for instance with N15, it was also possible to detect the lower M, breakdown products of the protein just as are also found in EBV-transformed B cell lines like X50-7 (Fig. 5; see also Wang et al., 1985; Rowe et al., 1987b).

However, interpretation of the gel in the lower M, region is complicated because the detection system again gives a signal at about 50K from immunoglobulin heavy chains present in the biopsy extracts. Taking the overall results from both MAbs CS1 to 4 and MAb S12 immunoblots, nine of the 24 fresh NPC biopsies which were demonstrably EBNA 1-positive also expressed detectable levels of LMP (see Table 1). In addition the nude mouse-passaged NPC line C15 was clearly LMP-positive when probed with CS1 to 4, although interestingly this protein was not detected by S12 (data not shown).

The LMP status of many of the NPC biopsies was also checked by immunoprecipitation with pooled MAbs CS1 to 4, the immunoprecipitates being run out on a gel and then blotted with the same CS1 to 4 reagent. None of the biopsies that were LMP-negative by immunoblotting gave any detectable LMP signal by immunoprecipitation. Fig. 6 presents the immunoprecipitation results from a panel of NPCs (N2 to N15) similar to those examined by immunoblotting in Fig. 5. Once again biopsies N2 and N15 were clearly LMP-positive and there was a very weak signal with N5, whilst the unusually low M, LMP in N14 had been obscured by the strong heavy chain signal from the MAb component of the immunoprecipitate. Again the nude mouse-passaged NPC line C15 was clearly LMP-positive.

Immunoblotting for lytic cycle antigens

Some of the human sera used in the earlier immunoblotting experiments to search for expression of EBNA proteins (for example serum RM, Fig. 3) were known also to contain
Fig. 5. Levels of LMP expression in NPC biopsies (N2, N5, N7, N11, N14, N15 and N16; lanes 3 to 9) as detected by SDS–PAGE and immunoblotting of tumour extracts (a) with a pool of four anti-LMP MAbs, CS1 to 4 and (b) with the anti-LMP MAb S12. Lane 1 represents the reference cell line X50-7 and lane 2 the carcinoma control CxCa.
antibodies to EBV lytic cycle antigens, yet the immunoblots never gave any indication that such antigens were being expressed in NPC biopsies.

In the final set of experiments we specifically addressed this question of lytic cycle antigen expression using a human serum (EE) whose reactivity in blotting is dominated by antibodies to the EA(D) complex (40K to 55K) and to the high (125K to 160K) and low (22K) Mr VCA components, and using the EA(D)-specific MAb R3. These two independent reagents gave very similar results, illustrated in Fig. 7 by an immunoblot obtained using the MAb R3. In the 40K to 55K region of the gel, where the various components of the EA(D) complex are to be found (compare the reference EBV producer cell line P3HR1 with the EBV-negative line BL2), there was no detectable EA(D) signal from any of the fresh NPC biopsies nor indeed from the nude mouse-passaged NPC line C15.

**DISCUSSION**

The question of virus gene expression in the biopsy cells of EBV-associated malignancies has only recently begun to be addressed at the protein level, taking advantage of monoclonal antibodies and of selected human sera with defined reactivities against viral antigens. Thus we have recently found that BL biopsy cells, and also many BL cell lines in early passage, seem to display an unusually restricted pattern of EBV latent gene expression with detectable levels of EBNA 1 but not of either EBNA 2 or LMP (Rowe et al., 1987a). This contrasts with the situation in in vitro transformed lymphoblastoid cell lines where the complete set of EBV latent gene products are constitutively expressed (reviewed by Dambaugh et al., 1986). The contrast between tumours and in vitro transformed cells in this case bears witness to the complex series of changes occurring at the cellular level during in vitro selection of a malignant BL cell clone. In the present work we show that a second EBV-associated malignancy, NPC, also shows an unusual
Fig. 7. Levels of EA(D) expression in NPC biopsies (N2, N5, N7, N11, N14, N15, N16; lanes 5 to 11) as detected by SDS-PAGE and immunoblotting of tumour extracts with the anti-EA(D) MAB R3.
Lane 1, P3HR1; lane 2, BL2; lane 3, CxCa; lane 4, C15 nude.

pattern of EBV latent proteins in which EBNA 1, and often LMP, are expressed in the absence of detectable amounts of any of the other EBNA species, EBNA 2, EBNA 3 and EBNA-LP.

As can be seen from Fig. 1, where NPC extracts were probed using a well characterized human serum Mo selectively reactive against EBNA 1, the level of detectable EBNA 1 protein per standard mass of biopsy material varied considerably between individual samples. One important factor influencing results here would be the relative proportions of malignant tissue and of normal stroma in these particular biopsy samples. The very small size of each snap-frozen sample made it necessary to commit the whole sample to protein analysis. Thus we have no direct measure of the cellular composition of the snap-frozen tissue, only the histological record from an adjacent piece of the biopsy specimen. In a minority of cases no EBNA 1 protein was detectable even though some malignant cells were present in the adjacent tissue reserved for histology; we interpreted this as signifying dilution of malignant cells below the sensitivity threshold of the immunoblotting assay. Preliminary screening of the 'EBNA 1-negative' biopsy extracts for other EBV proteins gave negative results and so these particular samples were excluded from the later series of experiments.

Many NPC biopsies gave two or more bands when immunoblotted with sera such as Mo (Fig. 1) which is operationally EBNA 1-specific in its reaction with B cell lines (see Rowe et al., 1987a). Antibody elution experiments formally proved, at least for biopsy N5, that these multiple bands were antigenically related to EBNA 1 (Fig. 2). Hence there may have been some degradation of EBNA 1 during storage and/or sample preparation, the precise size of the native EBNA 1 protein and of its breakdown products varying between different biopsies because each carried an individual EBV isolate. Note that when X50-7 cells were subjected to the same snap-freezing/extraction protocol as the NPC biopsies, this sometimes produced an additional EBNA 1-related band at 66K which was detected by Mo serum below the native EBNA 1 protein (see Fig. 1 and 2). At the same time, none of the other EBV latent gene products in X50-7 cells appeared to be affected by this experimental protocol (see Fig. 3 to 6).

Various human sera, selected for their strong reactivity against the other EBNA proteins, failed to detect EBNA 2, EBNA 3 or EBNA-LP in any of the 24 EBNA 1-positive NPC biopsies (e.g. Fig. 3). One possible explanation would be that these NPC-associated isolates of EBV encoded variant forms of the above antigens which were not recognizable by the sera employed in this work. However we find this extremely unlikely since these same sera consistently recognized the relevant EBNA species encoded by several different EBV isolates in spontaneous lymphoblastoid cell lines derived from the same area of Kenya (Maru) from which most of the NPC biopsies were obtained (Young et al., 1987). The EBNA 2-specific MAB PE2 is
particularly valuable in this regard since it recognizes the two alternative forms of this antigen, EBNA 2A and 2B, both of which are represented amongst Kenyan isolates of EBV (Young et al., 1987); none of the 24 NPC biopsies gave a specific EBNA 2 band with PE2 either by immunoblotting or by immunoprecipitation (Fig. 4). It is significant that all three nuclear antigens (EBNA 2, EBNA 3 and EBNA-LP) are detectable in snap-frozen biopsy tissue from another EBV-associated lesion, namely the lymphomas induced by experimental EBV infection of cotton-top tamarins (Finerty et al., unpublished data), so that the present negative results with NPC biopsy material are not an artefact of the experimental protocol but a real reflection of the type of virus–cell interaction that exists in NPC.

When the analysis of EBV latent gene expression was extended to LMP, differences between individual tumours were immediately apparent (Fig. 4c, 5, 6). Some gave no detectable expression with either of the specific MAbs whilst others were clearly LMP-positive. Note also that a positive LMP reaction was not restricted to those biopsies giving the strongest EBNA 1 signal; for instance biopsy N7 gave stronger EBNA 1 bands than N8 (Fig. 1) yet LMP was clearly detectable in N8 but not in N7 (Fig. 4c). It is therefore likely that these results reflect real differences in LMP expression between individual tumours and not just variation in the proportion of malignant cells in the biopsy population. What determines such differences remains to be seen but, given the known influence of cellular phenotype over EBV gene expression (Rowe et al., 1987a), it will be important in future to examine LMP status in the light of a more precise immunohistochemical definition of the epithelial tumour cell phenotype. The fact that many NPC biopsies express detectable levels of LMP is interesting since this protein has proven growth transforming ability, indeed the ability to bring about malignant cell transformation in a rodent cell system (Wang et al., 1985), and (at least in B lymphocytes) appears to interact with the cytoskeleton (Liebowitz et al., 1987). Further work will be required to compare tumours with quantitative differences in LMP expression by analysis at the single cell level. This will be of interest because, even in \textit{in vitro} transformed lymphoblastoid cell lines, there are marked differences between individual cells when stained for LMP by immunofluorescence (Rowe et al., 1987b).

The high titres of IgA antibodies to EA and VCA which are associated with NPC (Henle & Henle, 1976; Zeng, 1985) and the fact that such titres tend to reflect tumour load (Henle et al., 1977) would imply that the tumour itself is the source of these lytic cycle antigens. Indeed some NPC biopsies appear to express transcripts usually associated with entry of cells into the productive cycle (Raab-Traub et al., 1983; Tugwood et al., 1987) and the passage of NPC cells in nude mice in some cases leads to full virus replication in a small proportion of the cells (Trumper et al., 1977). However, in the present experiments we could find no detectable expression of lytic cycle antigens in NPC biopsies using either a selected human serum or the EA(D)-specific MAb R3 (Fig. 7). Note that the nude mouse-passaged C15 tumour also proved negative for lytic cycle antigens in our hands, and parallel RNA studies (N. Raab-Traub et al., unpublished data) have in fact confirmed the absence of any lytic cycle transcripts in this particular NPC line. If NPC cells in fresh biopsies are supporting EBV replication \textit{in vivo}, then this must be restricted to a very small proportion of the malignant population. There is as yet no direct evidence that such replication occurs at all.

This paper describes a type of EBV–cell interaction in NPC which is distinct from that seen in \textit{in vitro} transformed lymphoblastoid cell lines, and different from that seen in BL biopsies (Rowe et al., 1987a). The type of interaction observed does not seem to be influenced by the geographical origin of the tumours, since we have studied EBV-positive NPC biopsies from three different areas with dramatically different incidences of the tumour (Table 1). While this paper was in preparation we learnt that a similar study has been conducted on NPC biopsies from China (a high incidence area) as well as from North and East Africa with results which are essentially similar to our own (Fähræus et al., 1988). The unusual pattern of EBV latent gene expression in NPC is therefore a consistent feature of this tumour worldwide.

This work represents a first step in our attempts to understand the complex pathogenesis of NPC. It is not possible to judge the full significance of the present results until we know the pattern of EBV gene expression which occurs when the virus normally infects the
basal/suprabasal layers of pharyngeal epithelium in vivo (Young et al., 1986; Sixbey et al., 1987). This particular interaction, which must precede full replication of the virus in the outer more differentiated layers (Sixbey et al., 1983; Greenspan et al., 1985), is the one with which the EBV–epithelial cell interaction in NPC must be compared. It is tempting to suggest that down-regulation of the EBNA 2, EBNA 3 and EBNA-LP antigens is an important factor in the selection of a malignant NPC clone (Raab-Traub & Flynn, 1986) from the precursor pool of EBV-infected basal/suprabasal epithelial cells; indeed there would be analogies here with the evolution of EBV-positive BL (Rowe et al., 1987a). However, such down-regulation may be a general feature of virus gene expression in basal/suprabasal epithelium rather than a specific feature of NPC. Conversely it is possible that other EBV proteins, as yet unrecognized because their expression is restricted to epithelial cells, are being expressed alongside EBNA 1 and LMP in NPC cells and may even be contributing to the malignant process.

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EBV gene expression in NPC


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