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Original Paper

Antibodies to LMP2A/2B in EBV-carrying Malignancies

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Antibodies to the Epstein-Barr virus (EBV)-encoded membrane proteins, LMP2A and LMP2B, were assayed in 540 individuals, including 154 patients with nasopharyngeal carcinoma, 16 with African Burkitt's lymphoma, 113 with Hodgkin's disease, 14 with EBV-carrying gastric carcinoma, 14 with oral hairy leucoplakia (HIV+ patients), 37 with non-Hodgkin's lymphoma, 49 with tumours of the head/neck, 19 with infectious mononucleosis, 62 with chronic illnesses with EBV titres consistent with re-activations, and 62 healthy controls. A novel assay, mouse monoclonal enhanced indirect immunofluorescence assay (MIFA) was designed and used to test the sera for antibodies to the LMP2A and 2B proteins, expressed in human keratinocytes. Antibody to both LMP2A and LMP2B was strikingly specific to NPC. Virtually all (99 of 101) of the LMP2 antibody positive individuals were NPC patients, 95% of whom had antibodies that reacted both with the LMP2A- and LMP2B-transfected indicator cells, while the remaining 5% reacted only with the LMP2B expressing cells.

Key words: anti-LMP2, NPC, EBV malignancies
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INTRODUCTION

THREE DIFFERENT types of Epstein-Barr virus (EBV) latency have been identified, depending on the expression pattern of the growth transformation-associated EBNA (EBV-nuclear antigen) and LMP (latent membrane protein) complexes. EBV-carrying Burkitt's lymphoma (BL) cells express only EBNA 1, and the same is true for BL-derived cell lines that have maintained a representative BL cell phenotype. EBV transformed B-lymphocytes of non-neoplastic origin (lymphoblastoid cell lines, LCLs) and BL lines that have drifted to a more immunoblastic phenotype during serial *in vitro* propagation express six nuclear antigens (EBNA1–6), together with the three membrane proteins LMP1, LMP2A and LMP2B. Undifferentiated NPC (nasopharyngeal carcinoma) cells express EBNA1 but not EBNA2–6, and approximately 67% also express LMP1. In addition, LMP2A and LMP2B transcripts are regularly detected in the carcinoma cells, but there is no consensus as to their frequencies. Using nested PCR primers to increase assay sensitivity, we have recently found [1] that more than 90% of NPC biopsies express LMP1 and LMP2B, while LMP2A and LMP2B were co-expressed in only approximately 67% of the biopsies. Our findings were at variance to earlier reports [2–4] in which LMP2A transcripts were much more frequently detected than those of LMP2B or LMP1. There is even less information on the expression of the various LMPs in tumours. In NPC, LMP1 protein is present in only 67% of the tumours detected by immunoblotting [5,6]. There is no information on the *in situ* expressions of LMP2A and LMP2B proteins.

In 1993, Frech and colleagues, using LMP2A antigen (TP1) overexpressed in insect cells with baculovirus vectors, were able to detect LMP2A antibodies in approximately 40% of NPC patients of Mediterranean, Chinese and German ethnic origin. In contrast, all the HD (Hodgkin's disease), BL, IM (Infectious mononucleosis) and control patients they examined were LMP2A antibody negative [7]. In this study, we attempted to confirm those findings using LMP2A- and 2B-transfected human target cells to test 540 sera for LMP2-specific antibodies in patients representing a variety of EBV-associated diseases and controls.

MATERIALS AND METHODS

Cells

The human keratinocyte cell line FEP18-11 [8] was established by cotransfection of primary human foreskin keratinocytes with a plasmid clone of human papilloma virus 18. The coding sequences for the EBV LMP2A and LMP2B genes were derived from a LMP2A cDNA cloned in lambda gt10 [9]. The genes were cloned into the retroviral vector pLNPOX [10], which confers resistance to the drug G418. Recombinant retro-

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viruses were produced in PA 317 packaging cells [11], and were used to infect cultures of FEP18-11 cells at passage 21. Colonies, selected in 100 μg/ml (active concentration) of G418 (Sigma, St Louis, MO), were tested and monitored for LMP2A and LMP2B expression by cDNA–PCR [1].

The keratinocyte cultures were maintained in serum-free medium (GIBCO BRL 17005-018) supplemented with epidermal growth factor and bovine pituitary extract. For subcultivation, the monolayers were trypsinised and resuspended in a small volume of trypsin, which was removed from the cell sediment after brief centrifugation. The cells were then diluted in fresh medium to the desired cell density and grown for 3–5 days before the preparation of smears. The expression of LMP2A and 2B in the two transfectants was stable and could be maintained after many subcultivations in the non-selective medium used.

Sera

We tested sera collected from 540 individuals, including 88 NPC patients and 32 controls of Chinese, Malay and Kadazan ethnic groups in Malaysia (from the University of Malaya), 25 Swedish NPC patients and 41 U.S. NPC patients, 49 patients with malignancies of the head and neck regions (Karolinska Institute), 113 Hodgkin's disease patients (University of Uppsala), 14 HIV-1 positive patients with EBV-associated oral hairy leucoplakia (University of California, San Franciso, U.S.A.), 16 Ghanian BL (National Cancer Institute, Bethesda, U.S.A.) and 14 gastric carcinoma (Kuakini Medical Centre), 37 patients with non-Hodgkin's lymphoma (K. Merk, Karolinska Hospital) 19 with IM, 62 patients with undiagnosed chronic conditions, showing a serological picture consistent with EBV re-activations, and 30 Californian volunteer blood donors.

Frozen tissues from the 14 oral hairy leucoplakia patients were previously found to be positive for EBNA [12] by anticomplement immunofluorescence (ACIF), by the method of Reedman and Klein [13] modified as previously described [14]. Formalin-fixed, paraffin-embedded tissues from the 14 gastric carcinoma patients were positive, as previously reported, for the EB gene sequences by PCR and by *in situ* hybridisation using *Bam*HI-W probes [15].

EBV serology

Sera were assayed for IgG–viral capsid antigen (VCA) by indirect immunofluorescence according to the method of Henle and associates (1974) [16].

LMP2A and LMP2B serology

Cell smears were prepared by scraping the keratinocytes from the culture vessel, and these were then sedimented, washed once in buffered saline, resuspended at a density of 1×10^7/ml, deposited on to glass cover slips, air dried rapidly and fixed for 1 min in acetone. The negative control keratinocytes carrying the vector were routinely mixed 1:1 with the LMP2A or LMP2B transfectants prior to the preparation of the smears to provide internal control cells.

Antibodies monospecific to LMP2A or LMP2B transfectants were separately assayed for every serum using the three-step mouse antibody enhanced indirect immunofluorescence assay (MIFA).

MIFA assays

Heat-inactivated sera, diluted 1:10, were incubated for 30 min at 37°C with the fixed cell smears in the first step, followed by mouse monoclonal anti-human $IgG_{1,2,3}$ (ATCC HF6508) in the second, and FITC-goat anti-mouse in the final step. Neither the mouse monoclonal anti-human IgG nor the FITC-goat anti-mouse reacted with control or transfected human keratinocytes. A titre of $<1:10$ was considered negative.

RESULTS

MIFA assay and staining patterns

Initial attempts to detect antibodies to LMP2 using the standard indirect and anti-complement indirect immunofluorescence were unsuccessful. The former assay was insufficiently sensitive and the latter produced unacceptable background staining (data not shown). In the MIFA assay, the high specificity of the monoclonal mouse anti-human IgG as the second antibody and the additional amplification of the three-step procedure circumvented both problems.

Examples of anti-LMP2A and LMP2B fluorescent staining in the MIFA test are shown in Figure 1a–c. The intense, punctate patterns were distinct from the other known EBV-encoded antigens (VCA (viral capsid antigen), EA (early antigen) and EBNA). The cytoplasmic, punctate staining was characteristic

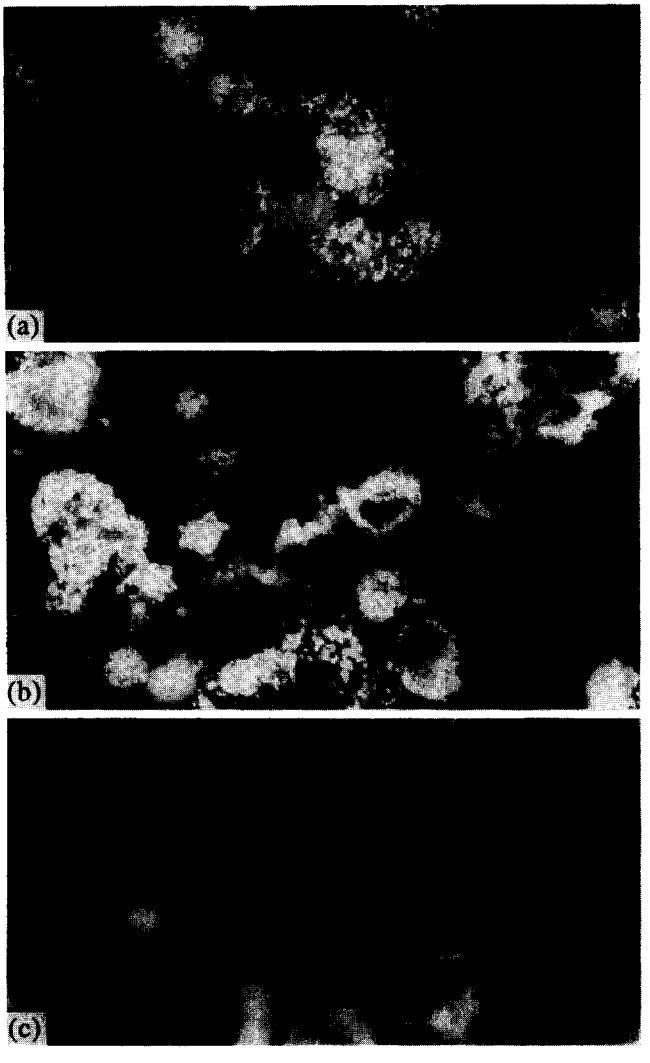

Figure 1. Staining patterns of LMP2A (a) and LMP2B (b) with NPC sera using the mouse monoclonal antibody-enhanced immunofluorescence assay (MIFA). (c) Negative control, human keratinocyte line FEP18-11 stained in parallel.

of both antigens, although LMP2B (Figure 1b) was noticeably coarser than LMP2A (Figure 1a) when observed under the microscope.

Differential reactivity of LMP2 sera

Of the 540 sera tested, 96 sera reacted with both LMP2A and LMP2B expressing indicator cells. Five additional (all NPC) sera reacted only with the LMP2B indicator cells, and did not stain the LMP2A-expressing cells. None of the sera tested reacted with LMP2A-expressing indicator cells alone. Due to the high degree of co-reactivity against LMP2A and LMP2B, the antibodies will be referred to collectively as anti-LMP2A/2B, even though every serum was tested separately for reactivity to LMP2A and LMP2B using the corresponding indicator cells.

Prevalence of anti-LMP2a and LMP2B

Table 1 summarises the prevalence of LMP2A/2B antibody among the 540 sera from the various patient groups, all of whom had moderate to high titre to IgG-VCA (IgG-VCA was not measured in those with head and neck cancer).

NPC. Almost all (99 of 101) of the LMP2A/2B antibody positive individuals were NPC patients. The prevalence of these antibodies were comparable among the three ethnic groups (Chinese, Malay and Kadazan) in Malaysia (70–78%). In comparison, 59% of the Californian NPC patients (80% of whom have Chinese ancestry) and 44% of the Swedish NPC patients were positive for anti-LMP2A/2B.

BL and other EBV-carrying tumours or lesions. We tested sera from 16 African Burkitt lymphoma patients. We also included 14 gastric carcinoma patients with known EBV DNA-carrying tumours and 14 HIV-1 infected patients with EBNA positive oral hairy leukoplakia. None of these 44 patients were positive for anti-LMP2A or LMP2B. In addition, we also tested 113 clinically diagnosed HD patients, all of whom were negative for anti-LMP2A or LMP2B. Among the 113 HD tumours, typed histologically according to the Rye system, there were 69 patients with nodular sclerosis subtypes, 33 with mixed cellularity, 6 with lymphocyte predominance, and 5 with lymphocyte depletion. 37 non-HD cases were also negative.

Table 1. Seroprevalence of anti-LMP2A/LMP2B

Patients	Number	VCA Geom. Mean Titre	Anti-LMP2A/LMP2B (+)	(−)	%
Chinese NPC	32	5232	25	7	78
Malay NPC	33	4609	23	10	70
Kadazan NPC	23	3903	16	7	70
California NPC	41	5034	24	17	59
Swedish NPC	25	1245	11	14	44
African Burkitts' lymphoma	16	11615	0	16	0
Gastric carcinoma	14	1159	0	14	0
Oral hairy leukoplakia	14	1345	0	14	0
Hodgkin's (Sweden)	113	878	0	113	0
Non-Hodgkin's disease	37	1714	0	37	0
Tumours of the head/neck	49	Not done	0	49	0
Chronic illness	62	11283	2	60	3
Infectious mononucleosis	19	510	0	19	0
Malaysian healthy controls	32	233	0	32	0
Californian volunteer blood donors	30	199	0	30	0

Tumours of the head–neck regions. 49 patients with diagnosed (non-NPC) tumours of the head and neck were tested. The tumour sites included the nasopharynx, larynx, tonsil, maxilla, thyroid, mandible, lip, mouth, middle ear and tongue. None were positive for anti-LMP2A/2B.

Conditions with exceptionally high EBV antibody titres. Of the 62 individuals with chronic illnesses selected for LMP2A/2B testing strictly for their unusually elevated EBV antibodies (IgG–VCA > 10240 or anti-EA > 320 or anti-EBNA > 2560), two were positive for anti-LMP2A/2B. All of the patients in this group were seen by their physicians for chronic conditions of unknown aetiologies.

IM patients and healthy controls. All of the Malaysian and Californian healthy controls (all IgG–VCA positive) were seronegative for LMP2A/2B, as were the patients with acute primary EBV infections (IgM–VCA positive).

DISCUSSION

Elevated antibody titres to the EBV antigens (VCA, EA) are frequently found in BL and NPC patients with EBV-carrying tumours [17]. The earlier systematic serological investigations showed, however, that no single antibody or immunoglobulin subclass against these antigens is tumour-specific. Hence, serology cannot be used to diagnose nor to differentiate clearly between cancer and normal patients.

The present investigation re-examines the seroprevalence of LMP2A/2B antibodies in EBV-associated malignancies, using the novel MIFA assay. This approach was chosen to exploit two features: the human keratinocytes represent human cells of epithelial lineage, which has not been routinely available for EBV serological assays, and the modified immunofluorescence assay we used, unlike enzyme immunoassays or immunoblotting, detect antigen epitopes in their native states. The large number of patients we investigated could be divided into four categories. The first were patients with documented EBV-carrying tumours or lesions; they included 14 cases of EBV genome-positive (by *in situ* hybridisation) gastric carcinoma and 14 EBNA-positive oral hairy leucoplakia. These patients were of particular interest as both conditions involved lesions of epithelial origin. The former is histologically and morphologically similar to the undifferentiated NPC; the latter involves terminally differentiated keratinocytes of the tongue epithelium and is found primarily in HIV-infected individuals [12]. The second group of patients included those with malignancies closely associated with EBV, namely NPC and African BL, and the third group included patients with tumours known to be associated with EBV, but to varying degrees. These included HD and patients with non-Hodgkin's lymphomas. The HD tumours included all four histological types. Although the EBV genome status of these HD patients are currently under investigation and not yet available, appropriately 50% of HD tumours examined by others with *in situ* hybridisation have been reported to be EBV genome-carrying [18]. Our last category included those with head and neck tumours other than NPC, healthy controls, infectious mononucleosis and patients with

EBV antibody profile consistent with reactivated infections. Depending on the clinical conditions, all the patient groups uniformly have elevated anti-VCA IgG titres, with geometric mean titres 2.5–50 times those of healthy controls.

The presence of anti-LMP2A/B did not correlate with anti-VCA IgG antibodies (Table 1). However, we found that LMP2A/2B antibodies were almost exclusively found in NPC patients. The association of anti-LMP2A/2B with NPC was not correlated to the patients' ethnic or geographic origin. The seroprevalence was highest (78%) among the Malaysian (Chinese and other ethnic groups) NPC, followed by Chinese American (60%) and was lowest in Swedish NPC patients (44%). We found a higher anti-LMP2A/B seroprevalence in NPC patients than Frech and associates who observed a mean prevalence of 38%, which increased to 50% after preselection of patients with stage 3 and 4 disease [7]. Hence, the quantitative difference observed between the two studies is unlikely to be due to the effects of staging; instead, they may be a reflection of the different assay systems used.

Two exceptional, non-NPC patients among our subjects had detectable anti-LMP2A/2B. Both had undiagnosed chronic conditions with highly elevated anti-EBV antibodies by routine EBV serology. Unfortunately, neither patient was available for further study and the clinical information was limited.

The absence of anti-LMP2A/2B is notable in several groups of patients. The antibodies were not detectable in Burkitt's lymphoma sera; this is in line with the known absence of LMP2A or 2B transcripts in the tumour. Surprisingly, the LMP2 antibodies were absent in all the 14 patients with EBV genome-positive undifferentiated gastric carcinoma. Given the histological similarity between the gastric and nasopharyngeal carcinoma, the difference seen in the LMP2 antibody profile is suggestive of differences in the mode of EBV gene expression in different epithelial tumours.

Transcripts of LMP2A and LMP2B are highly spliced and share homology in 7 of 8 exons. The 5′ exon 1 of LMP2A is missing from LMP2B, hence the latter antigenically represents a subset of the former. The native epitopes of LMP2B are contained in exons 2–7 and are recognised by sera from NPC patients [7]. Therefore, the exclusive reactivity observed in our study with the LMP2B indicator cells in 5% of the sera was puzzling. One explanation for our observations may be the presence of strain-specific LMP2A epitopes. Evidence for this was provided by Frech and colleagues [7] using hyperimmune rabbit antisera raised against LMP2A or two EBV strains. More recently, Busson and associates reported the existence of three patterns of sequence polymorphism in exon 1 of LMP2A [19].

The specificity of anti-LMP2A/2B in NPC is unique; no other EBV serological components, including IgA–VCA and –EA/D, show such a high degree of correlation with any of the EBV-associated tumours. The utility of LMP2A/2B antibodies in the diagnosis of EBV-associated NPC, therefore, needs to be evaluated clinically. The MIFA assay described by us allows such studies to be undertaken, and should also be of general utility in viral serology.

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