Jpn. J. Cancer Res. 83, 445–449, May 1992

Impaired Long-term T Cell Immunity to Epstein-Barr Virus in Patients with Nasopharyngeal Carcinoma

Shinji Tamura,[1,2] Atsushi Yamazaki,[1] Masaru Kunimoto,[1,2] Kiyoshi Takemura,[1] Toshihide Tabata,[2] Yorio Hinuma[1] and Osamu Yoshie[1,3]

[1]*Shionogi Institute for Medical Science, 2-5-1 Mishima, Settsu-shi, Osaka 566 and* [2]*Department of Otorhinolaryngology, Wakayama Medical College, 7 Bancho 27, Wakayama-shi, Wakayama 640*

The long-term T cell immunity to Epstein-Barr virus (EBV) is considered to play an important role in suppressing proliferation of EBV-infected B cells and outgrowth of EBV-associated tumors. It can be manifested and quantified by the EBV-induced focus regression assay. In the present study, we examined the strength of T cell immunity to EBV in patients with nasopharyngeal carcinoma (NPC) and other cancers originating from the head and neck region. In contrast to patients with other types of cancers, including EBV-negative NPC, patients with EBV-positive NPC were found to have a profound impairment in the long-term T cell immunity to EBV.

Key words: EBV — Nasopharyngeal carcinoma — Malignant lymphoma — T cell immunity

Epstein-Barr virus (EBV) is a ubiquitous lymphotropic human herpes virus which is carried in a state of latent infection by most normal individuals of all human populations. Paradoxically, however, EBV is also etiologically associated with endemic as well as sporadic forms of two human malignancies, Burkitt's lymphoma (BL) and undifferentiated nasopharyngeal carcinoma (NPC).[1] *In vitro,* EBV efficiently transforms B cells into indefinitely growing lymphoblastoid cell lines (LCLs) which produce the latency-associated viral proteins.[2] In healthy carriers, however, the growth of EBV-infected B cells is effectively suppressed by host immune surveillance. The importance of the immune surveillance is clearly demonstrated by the high incidences of EBV-positive B cell proliferative diseases and lymphomas associated with various immunodeficiency states.[3–5] While nonspecific effector cells, antibodies and cytokines no doubt contribute to inhibition of outgrowth of EBV-positive B cells,[6–8] EBV-specific HLA-restricted cytotoxic T cells are considered to play the major role in the immune surveillance.[9]

The long-term T cell immunity to EBV can be measured by the EBV-induced focus regression assay.[9] It has been established that the regression of EBV-induced transformation is exclusively seen in cultures of lymphocytes from seropositive donors, dependent on cell density and mediated by EBV-specific HLA-restricted immune memory T cells.[9] By using regression assay, it has been shown that holoendemic malarial infection, the long-suspected cofactor for endemic BL, suppresses EBV-specific T cell immunity.[10,11] A significant reduction in EBV-specific T cell immunity has also been demonstrated in endemic NPC patients by means of regression assay.[12,13] In the latter studies, however, it was not tested whether such a reduction in the regression activity is more frequently seen in NPC patients than those with other types of cancer. In the present study, therefore, we compared the long-term T cell immunity to EBV in Japanese patients having sporadic NPC with those having other types of cancer also originating from the same or a closely associated head and neck region by using the EBV-induced focus regression assay.

MATERIALS AND METHODS

All the patients were Japanese, seen at the hospital of Wakayama Medical College. A blood sample of each patient was obtained before starting therapy and the diagnosis was confirmed by histological examinations. Blood samples were also obtained from healthy donors. Serum antibody titers to VCA (virus capsid antigen), EA (early antigen) and EBNA (EBV-encoded nuclear antigen) were determined by the standard methods.[14] EBNA and LMP (latent membrane protein) were detected on frozen tissues by anti-complement immunofluorescence staining[15] and indirect immunoperoxidase staining using a monoclonal anti-LMP S12,[16] respectively. EBV-DNA was detected in the tissue samples by Southern blotting hybridization using the *Bam*HI W fragment of B95-8 EBV-DNA as the probe.[17]

The level of T cell-mediated immunity to EBV in peripheral blood lymphocyte (PBL) samples was measured by means of focus regression assay as described previously[18] with slight modifications. In brief, peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples by isopycnic centrifugation on Ficoll-Paque (Pharmacia). Cells adherent to the plastic surface were removed by cultivation at 37°C for 2 h to

[3] To whom all correspondence should be addressed.

overnight. Non-adherent cells (mainly PBLs) were pelleted, and 1.6×10^6 cells were incubated in 0.5 ml of EBV stock derived from B95-8 at 37°C for 1 h. We used a single lot of the B95-8 culture supernatant with a 50% transforming titer of $10^{3.5}$/ml as the EBV source throughout the present study. Cells were washed twice with culture medium, and resuspended in 2 ml of RPMI-1640 supplemented with 10% fetal bovine serum. After two-fold serial dilutions, cells were seeded into round-bottomed 96-well type microtest plates (150 μl/well) at 8×10^5, 4×10^5, 2×10^5, 1×10^5, 5×10^4, 2.5×10^4, 1.25×10^4 and 6.25×10^3 cells/ml, using six replicate wells per cell concentration. After about 40 days, outgrowth of immortalized cells in each well was determined under a microscope. The regression index, which was the initial cell concentration ($\times10^{-4}$) per ml required to achieve 50% incidence of regression in EBV-infected PBL cultures, was calculated by the Reed and Muench method; the higher the regression index, the lower the frequency of EBV-specific cytotoxic T cells in the PBL population. The statistical analysis was done by applying the Mann-Whitney U test.

RESULTS

The regression assay was carried out with PBL samples obtained from healthy donors and patients with NPC and other cancers originating from the head and neck region. The results are summarized in Table I. PBL samples derived from four seropositive healthy donors exhibited high regression activities with the average regression index of 8.1. On the other hand, PBL samples obtained from seronegative healthy donors showed no regression activity at all (RI >160). These results confirmed that the regression activity was dependent on previous sensitization to EBV.[9] All the patients in the present study were seropositive. In the case of PBL samples obtained from NPC patients, the regression activity was quite variable, ranging from 6.0 to >160. NPC of two patients was EBV-negative squamous cell carcinoma. The values of these patients (6.2 and 6.0) were quite similar to those of normal seropositive donors. In sharp contrast, the regression activities of four other patients who had EBV-positive non-keratinizing or undifferentiated NPC were quite low, the average regression index being >120. We also examined the regression activity of four patients suffering from other squamous cell carcinomas originating from the head and neck region. These tumors are not associated with EBV. Their regression activities were similar to those of normal seropositive donors, the average regression index being 7.1. Collectively, the dramatic increases in the regression index in EBV-positive NPC patients were highly significant ($P<0.05$), whereas patients with EBV-negative NPC or other types of cancer also originating in the head and neck region did not show any significant difference in the regression index from healthy seropositive donors.

We also studied seven patients with malignant lymphomas developing from the head and neck region. Some Hodgkin's disease and other types of malignant lymphoma are now known to be infected with EBV.[19,20] Tumors of two patients were found to be EBV-positive by immunostaining for EBNA and/or detection of EBV DNA by Southern blotting hybridization. (Detailed phenotypic and other analyses of EBV-positive lymphomas will be published elsewhere.) Five patients whose tumors were EBV-negative again showed regression activities comparable to those of normal seropositive donors. The average regression index was 8.2. One patient with EBV-positive non-Hodgkin's lymphoma of diffuse, large cell type had a normal level of regression activity (RI=8.1), whereas one with EBV-positive non-Hodgkin's lymphoma of diffuse, mixed cell type showed a very low regression activity with a regression index of 64, which is a value similar to those of EBV-positive NPC patients.

DISCUSSION

Certain human malignancies are now known to be etiologically associated with viruses. These include BL and NPC associated with EBV,[1] cervical carcinoma associated with HPV[21,22] and adult T-cell leukemia (ATL) associated with HTLV-1.[23] These virus-associated human malignancies often express virus-encoded antigens which may function as tumor-specific antigens and may trigger attack from the host immune surveillance system. Therefore, these virus-associated malignancies provide tumor models in which interactions between tumor cells and the host immune system can be studied.[24] In the present study, we have demonstrated that a dramatic reduction in the long-term T cell immunity to EBV is only seen in patients with EBV-positive NPC and not in those with other types of cancer even originating in the same or closely related anatomical regions. Such strong and consistent impairments in T cell immunity to EBV have only been reported in recipients of organ transplants treated with immunosuppressive drugs,[25] patients with AIDS[26,27] and patients with ATL.[28] We also found reduced T cell immunity to EBV in one of two patients with EBV-positive malignant lymphoma, but the significance of this observation remains to be established.

In the case of BL, tumor cells usually produce only EBNA-1, which is essential for maintenance of episomal growth of EBV. Other latent gene products which are present in EBV-induced lymphoblastoid cell lines and most of which have been shown to serve as good targets for cytotoxic T cells[29–32] are not produced.[2] The mecha-

Table I. Summary of Serum Anti-EBV Titers, Immunohistological Detection of EBNA and LMP, Hybridization Detection of EBV-DNA and Regression Index

Donor	Age	Diagnosis	Serum antibody titer					Antigen in tumor		EBV DNA in tumor	RI
			VCA		EA		EBNA				
			IgG	IgA	IgG	IgA		EBNA	LMP		
Seronegative donors											
N.N.	8	normal	<10	<10	<10	<10	<10	ND	ND	ND	>160
N.N.	10	normal	<10	<10	<10	<10	<10	ND	ND	ND	>160
Y.Y.	19	normal	<10	<10	<10	<10	<10	ND	ND	ND	>160
Seropositive donors											
Y.U.	10	normal	160	<10	<10	<10	80	ND	ND	ND	6.6
S.T.	27	normal	80	<10	<10	<10	80	ND	ND	ND	7.1
J.Y.	30	normal	80	<10	<10	<10	80	ND	ND	ND	6.4
T.J.	30	normal	40	<10	<10	<10	40	ND	ND	ND	12
Patients with EBV-negative NPC											
N.S.	43	NPC SCC (IV)	40	<10	<10	<10	80	−	ND	−	6.2
Y.N.	74	NPC SCC (IV)	640	80	<10	<10	20	−	−	−	6.0
Patients with EBV-positive NPC											
K.H.	48	NPC NKC (IV)	320	40	80	<10	160	+	+	+	>160
T.N.	59	NPC UC (III)	5120	320	320	<10	320	+	+	+	62
H.Y.	63	NPC NKC (IV)	320	<10	160	<10	40	+	−	+	>160
K.O.	70	NPC UC (III)	1280	80	320	40	80	+	−	ND	80
Patients with other carcinomas											
T.T.	46	LC SCC	80	<10	<10	<10	160	ND	ND	ND	3.5
S.T.	52	MC SCC	20	<10	<10	<10	80	ND	ND	ND	10
F.N.	74	LC SCC	160	40	NS	<10	160	ND	ND	ND	7.1
T.N.	74	EC SCC	160	<10	<10	<10	40	ND	ND	ND	7.8
Patients with EBV-negative lymphomas											
T.Y.	61	NHL DM (II)	80	<10	<10	<10	40	−	−	−	7.6
M.O.	69	HD LP (II)	80	<10	<10	<10	80	−	−	−	15
T.T.	70	NHL ALC (II)	80	<10	<10	<10	<10	−	−	−	3.2
F.N.	70	NHL DL (I)	320	<10	<10	<10	80	−	−	−	7.1
K.M.	85	NHL DL (II)	320	<10	40	<10	80	−	−	−	7.8
Patients with EBV-positive lymphomas											
Y.K.	52	NHL DL (I)	640	<10	10	<10	40	+	−	+	8.1
H.I.	69	NHL DM (II)	320	20	20	<10	80	−	−	+	64

Abbreviations are as follows. RI, regression index; VCA, virus capsid antigen; EA, early antigen; EBNA, EBV nuclear antigen; SCC, squamous cell carcinoma; NKC, non-keratinizing carcinoma; UC, undifferentiated carcinoma; LC, laryngeal carcinoma; MC, maxillary carcinoma; EC, ear carcinoma; NHL, non-Hodgkin's lymphoma; HD, Hodgkin's disease; DM; diffuse-mixed cell type; LP, lymphocytic predominance type; ALC, anaplastic large cell type; DL, diffuse-large cell type; ND, not done; NS, nonspecific. The number in the parenthesis indicates the stage.

nism of restricted gene expression in BL is largely unknown, but Sample *et al.*[33] have recently demonstrated a hitherto unrecognized promoter unique for EBNA-1 transcription. The suppression of EBV latent genes in BL most likely has a strong selective advantage during multi-step tumor progression *in vivo* under the influence of immune surveillance by EBV-specific cytotoxic T cells. Recent studies have also shown that BL cells evade immune surveillance by down-regulation of virus-induced lymphocyte-detected membrane antigen (LYDMA) and sometimes even HLA class I antigens, both of which are essential for specific recognition by cytotoxic T cells.[34-36] Furthermore, down-regulation of certain cell adhesion molecules seen in BL cells also contributes to resistance to cytotoxic T cells.[37,38] These results collectively support the importance of T cell surveillance during *in vivo* evolution of BL. In the case of NPC, besides EBNA-1 about 50% of tumors were shown

to express LMP,[2] which is a membrane protein recognized by cytotoxic T cells.[29,31] We also detected LMP expression in two out of four EBV-positive NPC cases in the present study. The reason for or the mechanism of the persistent expression of LMP in about 50% of NPC is not known, but these and other data suggest that there are important differences in the regulation of EBV gene expression between NPC and BL.[39,40] NPC patients in fact have a profile of serological responses to EBV-encoded antigens distinct from that of BL patients.[14] The anergy in T cell immunity to EBV shown by previous studies[12,13] and by the present study, therefore, might be advantageous for the development of NPC. It is not known at present how the T cell immunity to EBV could be suppressed in NPC patients. It may be due to a persistent overload of EBV, induction of antigen-specific suppressor T cells and/or other mechanisms of immune suppression. Further studies will be necessary for a more comprehensive understanding of the interaction between tumor cells and the host's immune surveillance in NPC patients. Such studies may eventually suggest novel diagnostic and therapeutic approaches to NPC.

ACKNOWLEDGMENTS

We thank Drs. D. A. Thorley-Lawson and P. J. Farrell for providing S12 and the BamHI W fragment of B95-8 EBV-DNA, respectively. We also thank Dr. T. Katsuki for valuable advice.

(Received December 27, 1991/Accepted February 19, 1992)

REFERENCES

1) Henle, W. and Henle, G. Epstein-Barr virus and human malignancies. *Adv. Viral Oncol.*, **5**, 201–238 (1985).
2) Klein, G. Viral latency and transformation: the strategy of Epstein-Barr virus. *Cell*, **58**, 5–8 (1989).
3) Purtilo, D. T., Tatsumi, E., Manolov, G., Manolova, Y., Harada, S., Lipscomb, H. and Krueger, G. Epstein-Barr virus as an etiological agent in the pathogenesis of lymphoproliferative and aproliferative diseases in immune deficient patients. *Int. Rev. Exp. Pathol.*, **27**, 113–183 (1985).
4) List, A. F., Greco, F. A. and Vogler, L. B. Lymphoproliferative diseases in immunocompromised hosts: the role of Epstein-Barr virus. *J. Clin. Oncol.*, **5**, 1673–1689 (1987).
5) Ernberg, I. Epstein-Barr virus and acquired immunodeficiency syndrome. *Adv. Viral Oncol.*, **8**, 203–217 (1989).
6) Thorley-Lawson, D. A. The transformation of adult but not newborn human lymphocytes by Epstein-Barr virus and phytohemagglutinin is inhibited by interferon: the early suppression by T cells of Epstein-Barr infection is mediated by interferon. *J. Immunol.*, **126**, 829–833 (1981).
7) Kaplan, J. and Shope, T. C. Natural killer cells inhibit outgrowth of autologous Epstein-Barr virus-infected B lymphocytes. *Nat. Immun. Cell Growth Regul.*, **4**, 40–47 (1985).
8) Kure, S., Tada, K., Wada, J. and Yoshie, O. Inhibition of Epstein-Barr virus infection *in vitro* by recombinant human interferons α and γ. *Virus Res.*, **5**, 377–390 (1986).
9) Rickinson, A. B. and Moss, D. J. Epstein-Barr virus-induced transformation: immunological aspects. *Adv. Viral Oncol.*, **3**, 213–238 (1983).
10) Moss, D. J., Burrows, S. R., Castelino, D. J., Kane, R. G., Pope, J. H., Rickinson, A. B., Alpers, M. P. and Heywood, P. F. A comparison of Epstein-Barr virus-specific T-cell immunity in malaria-endemic and -nonendemic regions of Papua New Guinea. *Int. J. Cancer*, **31**, 727–732 (1983).
11) Whittle, H. C., Brown, J., Marsh, K., Greenwood, B. M., Seidelin, P., Tighe, H. and Wedderburn, L. T-cell control of Epstein-Barr virus-infected B cells is lost during *P. falciparum* malaria. *Nature*, **312**, 449–450 (1984).
12) Chan, S. H. and Chew, T. S. Lack of regression in Epstein-Barr virus infected leucocyte cultures of nasopharyngeal carcinoma patients. *Lancet*, **ii**, 1353 (1981).
13) Moss, D. J., Chan, S. H., Burrows, S. R., Chew, T. S., Kane, R. G., Staples, J. A. and Kunaratnam, N. Epstein-Barr virus specific T-cell response in nasopharyngeal carcinoma patients. *Int. J. Cancer*, **32**, 301–305 (1983).
14) Henle, W. and Henle, G. Immunology of Epstein-Barr virus. *Compr. Virol.*, **1**, 209–252 (1982).
15) Reedman, B. M. and Klein, G. Cellular localization of an Epstein-Barr virus (EBV)-associated complement-fixing antigen in producer and non-producer lymphoblastoid cell lines. *Int. J. Cancer*, **11**, 499–520 (1973).
16) Mann, K. P., Staunton, D. and Thorley-Lawson, D. A. Epstein-Barr virus-encoded protein found in plasma membranes of transformed cells. *J. Virol.*, **55**, 710–720 (1985).
17) Maniatis, T., Fritsch, E. F. and Sambrook, J. "Molecular Cloning: A Laboratory Manual" (1985). Cold Spring Harbor Laboratory, New York.
18) Rickinson, A. B., Moss, D. J., Wallace, L. E., Rowe, M., Misko, I. S., Epstein, M. A. and Pope, J. H. Long-term T-cell-mediated immunity to Epstein-Barr virus. *Cancer Res.*, **41**, 4216–4221 (1981).
19) Weiss, L. M., Movahed, L. A., Warnke, R. A. and Sklar, J. Detection of Epstein-Barr viral genomes in Reed-Sternberg cells of Hodgkin's disease. *N. Engl. J. Med.*, **320**, 502–506 (1989).
20) Harabuchi, Y., Yamanaka, N., Kataura, A., Imai, S., Kinoshita, T., Mizuno, F. and Osato, T. Epstein-Barr virus in nasal T-cell lymphomas in patients with lethal midline granuloma. *Lancet*, **335**, 128–130 (1990).
21) zur Hausen, H. Papillomaviruses in anogenital cancer as a model to understand the role of viruses in human cancers.

Cancer Res., **49**, 4677–4681 (1989).
22) Maki, H., Saito, S., Ibaraki, T., Ichijo, M. and Yoshie, O. Use of universal and type-specific primers in the polymerase chain reaction for the detection and typing of genital human papillomaviruses. *Jpn. J. Cancer Res.*, **82**, 411–419 (1991).
23) Yamamoto, N. and Hinuma, Y. Viral aetiology of adult T-cell leukaemia. *J. Gen. Virol.*, **66**, 1641–1660 (1985).
24) Klein, G. and Klein, E. Immune surveillance against virus-induced tumors and nonrejectability of spontaneous tumors: contrasting consequences of host versus tumor evolution. *Proc. Natl. Acad. Sci. USA*, **74**, 2121–2125 (1977).
25) Yao, Q. Y., Rickinson, A. B., Gaston, J. S. H. and Epstein, M. A. *In vitro* analysis of the Epstein-Barr virus: host balance in long-term renal allograft recipients. *Int. J. Cancer*, **35**, 43–49 (1985).
26) Birx, D. L., Redfield, R. R. and Tosato, G. Defective regulation of Epstein-Barr virus infection in patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related disorders. *N. Engl. J. Med.*, **314**, 874–879 (1986).
27) Ragona, G., Sirianni, M. C., Soddu, S., Vercelli, B., Sebastiani, G., Piccoli, M. and Aiuti, F. Evidence for disregulation in the control of Epstein-Barr virus latency in patients with AIDS-related complex. *Clin. Exp. Immunol.*, **66**, 17–24 (1986).
28) Katsuki, T., Yamaguchi, K., Matsuoka, Y. and Hinuma, Y. Impairment of T-cell control of Epstein-Barr virus infected B-cells in patients with adult T-cell leukemia. *AIDS Res.*, **2** (Suppl. 1), 125–130 (1986).
29) Thorley-Lawson, D. A. and Israelsohn, E. S. Generation of specific cytotoxic T cells with a fragment of the Epstein-Barr virus-encoded p63/latent membrane protein. *Proc. Natl. Acad. Sci. USA*, **84**, 5384–5388 (1987).
30) Moss, D. J., Misko, I. S., Burrows, S. R., Burman, K., McCarthy, R. and Sculley, T. B. Cytotoxic T-cell clones discriminate between A- and B-type Epstein-Barr virus transformants. *Nature*, **331**, 719–721 (1988).
31) Murray, R. J., Wang, D., Young, L. S., Wang, F., Rowe, M., Kieff, E. and Rickinson, A. B. Epstein-Barr virus-specific cytotoxic T-cell recognition of transfectants expressing the virus-coded latent membrane protein LMP. *J. Virol.*, **62**, 3747–3755 (1988).
32) Burrows, S. R., Sculley, T. B., Misko, I. S., Schmidt, C. and Moss, D. J. An Espstein-Barr virus-specific cytotoxic T cell epitope in EBV nuclear antigen 3 (EBNA 3). *J. Exp. Med.*, **171**, 345–349 (1990).
33) Sample, J., Brooks, L., Sample, C., Young, L., Rowe, M., Gregory, C., Rickinson, A. and Kieff, E. Restricted Epstein-Barr virus protein expression in Burkitt lymphoma is due to a different Epstein-Barr nuclear antigen 1 transcriptional initiation site. *Proc. Natl. Acad. Sci. USA*, **88**, 6343–6347 (1991).
34) Rooney, C. M., Rowe, M., Wallace, L. E. and Rickinson, A. B. Epstein-Barr virus-positive Burkitt's lymphoma cells not recognized by virus-specific T-cell surveillance. *Nature*, **317**, 629–631 (1985).
35) Rowe, D. T., Rowe, M., Evan, G. I., Wallace, L. E., Farrell, P. J. and Rickinson, A. B. Restricted expression of EBV latent genes and T-lymphocyte-detected membrane antigen in Burkitt's lymphoma cells. *EMBO J.*, **5**, 2599–2607 (1986).
36) Masucci, M. G., Torsteinsdottir, S., Colombani, J., Brautbar, C., Klein, E. and Klein, G. Down-regulation of class I HLA antigens and of Epstein-Barr virus-encoded latent membrane protein in Burkitt lymphoma lines. *Proc. Natl. Acad. Sci. USA*, **84**, 4567–4571 (1987).
37) Gregory, C. D., Murray, R. J., Edwards, C. F. and Rickinson, A. B. Downregulation of cell adhesion molecules LFA-3 and ICAM-1 in Epstein-Barr virus-positive Burkitt's lymphoma underlines tumor cell escape from virus-specific T cell surveillance. *J. Exp. Med.*, **167**, 1811–1824 (1988).
38) Billaud, M., Rousset, F., Calender, A., Cordier, M., Aubry, J.-P., Laisse, V. and Lenoir, G. M. Low expression of lymphocyte function-associated antigen (LFA)-1 and LFA-3 adhesion molecules is a common trait in Burkitt's lymphoma associated with and not associated with Epstein-Barr virus. *Blood*, **75**, 1827–1833 (1990).
39) Gilligan, K. J., Sato, H., Rajadurai, P., Busson, P., Young, L., Rickinson, A., Tursz, T. and Raab-Traub, N. Novel transcription from the Epstein-Barr virus terminal EcoRI fragment, DIJhet, in a nasopharyngeal carcinoma. *J. Virol.*, **64**, 4948–4956 (1990).
40) Gilligan, K. J., Rajadurai, P., Lin, J.-C., Busson, P., Abdel-Hamid, M., Prasad, U., Tursz, T. and Raab-Traub, N. Expression of the Epstein-Barr virus BamHI A fragment in nasopharyngeal carcinoma: evidence for a viral protein expressed *in vivo*. *J. Virol.*, **65**, 6252–6259 (1991).