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Detection of MMP and oxidized phospholipids

The MDMs were first treated for 15 min with rVpr and then with 5 μg/ml Rhodamine 123 (Sigma Chemical Co.) for another 15 min. Then, cells were washed with cold PBS and subjected to analysis of MMP by a LSC system (Olympus, Tokyo, Japan). For immunostaining of OxPC, the cells were washed with cold PBS and fixed using 4% PFA. After fixation, the cells were blocked with 3% BSA containing PBS. Thereafter, the OxPC-specific mAb DLH3 [33, 34] was treated. As a control, a mAb of isotyped IgM (Rockland Immunochemicals, Philadelphia, PA, USA) was used. Then, an Alexa-555-labeled anti-mouse IgM (Invitrogen) as a secondary antibody was treated for 1 h at room temperature. Nuclear DNA was stained with Hoechst 33342 (Invitrogen).

For the blocking experiments, we cultured the MDMs with control IgM or DLH3 (5 nM) for 20 min and then added rVpr and the primary antibodies (20 nM) and cultured these for 30 min. We then collected IL-6 mRNA and analyzed these molecules using qPCR. For the immunostaining experiments, we fixed the cells with cold methanol and blocked them with 5% BSA containing PBS. Thereafter, the anti-C/EBP-β antibody (Cell Signaling Technology) was used as a primary antibody, and Alexa-546-labeled anti-rabbit IgG (Invitrogen) was used as a secondary antibody. Nuclear DNA was stained with Hoechst 33342 (Invitrogen).

Biacore system

The possible interaction of rVpr and a rTLR4 protein was examined by using the Biacore J system (GE Healthcare). rVpr were coupled with sensor chip CM5 (GE Healthcare; BR-1005-30) by the amine coupling kit (GE Healthcare; BR-1000-10), and soluble TLR4, which was expressed by the baculovirus system and purified as described [35], was used as an analyte. Binding analysis of binding was done, following the manufacturer's instructions.

Statistical analysis

Data were generated from at least three replicate experiments. The statistical analysis was conducted by the Mann-Whitney U test. A *P* value of <0.05 was considered to be statistically significant.

RESULTS

IL-6 production from MDMs is stimulated by rVpr

We reported previously that the maximum concentration of Vpr present in patient plasma was ~5 ng/ml (0.3 nM) [13]. Thus, we thought it reasonable to investigate the biological activity of exogenous rVpr added at the ng/ml level. We first examined the activity of highly purified rVpr, which was prepared by affinity column chromatography using a mAb against rVpr (8D1; **Fig. 1A**). Following the addition of purified rVpr (Supplemental Fig. 1, A and B) to latently infected U1 cells [4], remarkable viral reproduction was observed when the cells were cocultured with PBMCs (*P*<0.05; Fig. 1B). In contrast, rVpr alone induced no reproductive activity in U1 cells (Fig. 1B, second column from the left, and Supplemental Fig. 2). CM prepared from PBMCs cultured with rVpr for 2 days also showed viral reproductive activity (Fig. 1C), suggesting that rVpr stimulates PBMCs to generate a humoral factor(s) that induces viral reproduction in U1 cells. Note that even 3 or 6 ng/ml rVpr, which is comparable with the concentration present in the plasma of HIV-1-positive patients, induced viral reproduction (Fig. 1C). To characterize the factors responsible for viral production, CM was prepared from the PBMCs of three healthy volunteers and subjected to protein array analysis (Supplemental Fig. 3). Among the factors tested, IL-6 was selected as a candidate, as its production was reproducibly correlated with the level of rVpr-induced viral production. Consistent with this, anti-IL-6 antibodies blocked the viral induction activity of the CM (Supplemental Table 1), suggesting that the factor responsible for the rVpr-induced reactivation of viral production was IL-6.

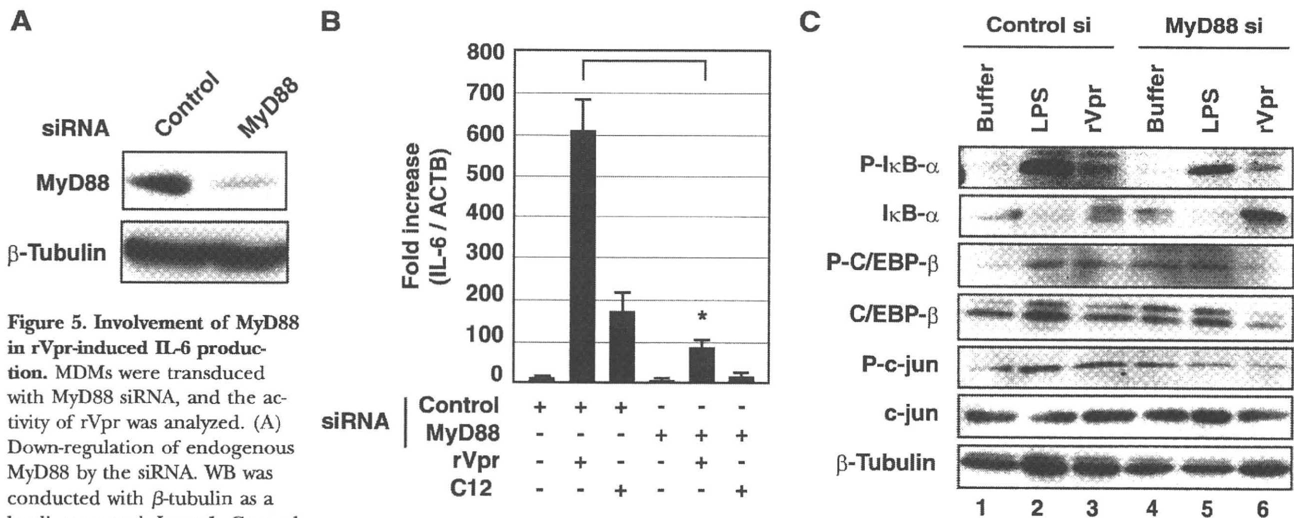

Figure 5. Involvement of MyD88 in rVpr-induced IL-6 production. MDMs were transduced with MyD88 siRNA, and the activity of rVpr was analyzed. (A) Down-regulation of endogenous MyD88 by the siRNA. WB was conducted with β-tubulin as a loading control. Lane 1, Control siRNA; lane 2, MyD88 siRNA. (B) The production of IL-6 induced by rVpr was abrogated by the down-regulation of MyD88. Following the introduction of MyD88 siRNA, MDMs were treated with rVpr or ΔC12 at 10 ng/ml. IL-6 production was then measured by RT-qPCR. *, $P < 0.05$. (C) Involvement of the MyD88-independent pathway in rVpr-induced IL-6 production. After the introduction of MyD88 siRNA (si), MDMs were treated for 40 min with 10 ng/ml rVpr or 10 pg/ml LPS, and the phosphorylation of each transcription factor was analyzed by WB. Lanes 1–3 represent control siRNA-treated cells, and lanes 4–6 represent cells treated with MyD88 siRNA. Lanes 1 and 4, Buffer control; lanes 2 and 5, LPS; lanes 3 and 6, rVpr. β-Tubulin was included as a loading control. MyD88 siRNA inhibited LPS-induced IL-6 production. LPS was added at 10 pg/ml for 3 h, and the expression of IL-6 in MDMs was analyzed by RT-qPCR. The data were obtained from three independent experiments. The values are expressed as the mean ± SD.

TABLE 1. Effects of Anti-TLR4 on Vpr-Induced IL-6 Production

Stimulators	W/o IgG	IgG		
		Control	α-TLR2	α-TLR4
None	1.0 ± 0.83	2.3 ± 1.38	2.2 ± 0.34	0.5 ± 0.32
rVpr	40.5 ± 10.8	37.0 ± 6.75[a]	40.1 ± 18.4	19.9 ± 9.78[a]

(Fold increase). [a]$P < 0.05$. Each antibody was added to MDMs cultured in the presence of rVpr, and then, IL-6 in the supernatant was measured by IL-6 ELISA. Of note, α-TLR4 inhibited IL-6 production from MDMs stimulated by LPS. W/o, Without.

To confirm that IL-6 was induced by rVpr, we measured IL-6 directly. Analysis by ELISA and RT-qPCR revealed that the addition of rVpr to MDMs induced the production of IL-6 significantly ($P<0.05$; Fig. 1, D and E). Additionally, we found that a comparable amount of rIL-6 induced viral reproduction in a dose-dependent manner (Fig. 1F). Next, to identify the cells responsible for rVpr-induced IL-6 production, we performed fractionation experiments using an antibody against CD14, a monocyte marker. We first removed all CD14-positive cells from the PBMCs. The recovered cells (Supplemental Fig. 4) were then cultured in the presence of rVpr. As shown in Figure 1G, no IL-6 production was observed following the addition of rVpr to the CD14-negative cell fraction ($P<0.05$; Fig. 1G). In contrast, the CD14-positive cells recovered from the MDMs showed vigorous IL-6 production in response to rVpr ($P<0.05$; Fig. 1H). These data indicate that rVpr-induced IL-6 production is dependent on monocytes/MDMs and that IL-6 is a mediator of rVpr-induced viral reproduction.

As our findings for rVpr were similar to those obtained with LPS, we performed additional experiments. First, we detected no LPS using the LAL test, a highly sensitive method with a detection limit of 0.001 EU/ml LPS (Supplemental Fig. 1A). Second, the activity of rVpr was abolished completely by boiling for 5 min, whereas that of LPS was reduced only partially following the same procedure (Supplemental Fig. 1B). Additionally, Vpr-induced IL-6 production was eliminated by pretreatment with a mAb against Vpr but not by control IgG (Supplemental Fig. 5). These results support the idea that the activation of MDMs by IL-6 represents authentic Vpr activity. We therefore sought to further characterize the signaling cascades induced by rVpr.

NF-κB activation is required for rVpr-induced IL-6 production

The *IL-6* promoter contains binding sites for four transcription factors: NF-κB, C/EBP-β, CREB, and AP-1. To identify the transcription factor(s) essential for rVpr-induced *IL-6* mRNA production, we performed a promoter assay using firefly *luciferase* as a reporter gene and observed that rVpr increased the activity of the *IL-6* promoter (**Fig. 2A**). In contrast, mutations in each IL-6 transcription factor-binding site markedly diminished *IL-6* promoter activity ($P<0.05$; Fig. 2A), suggesting that all of the transcription factors examined are required for rVpr-induced IL-6 production.

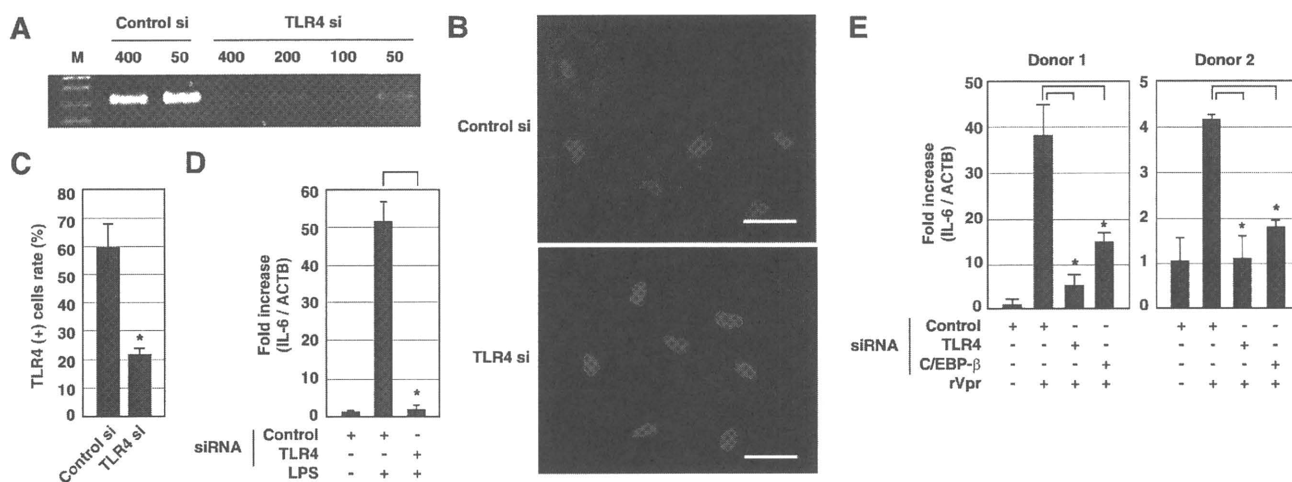

Figure 6. Involvement of TLR4 in rVpr-induced IL-6 production. MDMs were transfected with TLR4 siRNA, and their reactivity to rVpr was analyzed. The knockdown of endogenous TLR4 was confirmed by RT-PCR (A) and immunofluorescent staining (B; red, TLR4; blue, nucleus; original scale bars, 10 μm). M, marker. (C) TLR4 siRNA decreased the number of TLR4-positive cells, and the positive cells in the samples with the control and TLR4 siRNAs were counted. (D and E) The response to LPS or rVpr by cells with down-regulated TLR4. MDMs induced with TLR4 siRNA were treated with 10 pg/ml LPS (D) or 10 ng/ml rVpr (E) for 3 h. Following siRNA treatment, *IL-6* mRNA expression was analyzed by RT-qPCR. Responsiveness to rVpr was examined in two healthy donors (Donors 1 and 2). The data were obtained from three independent experiments. The values are expressed as the mean ± SD. *, $P < 0.05$.

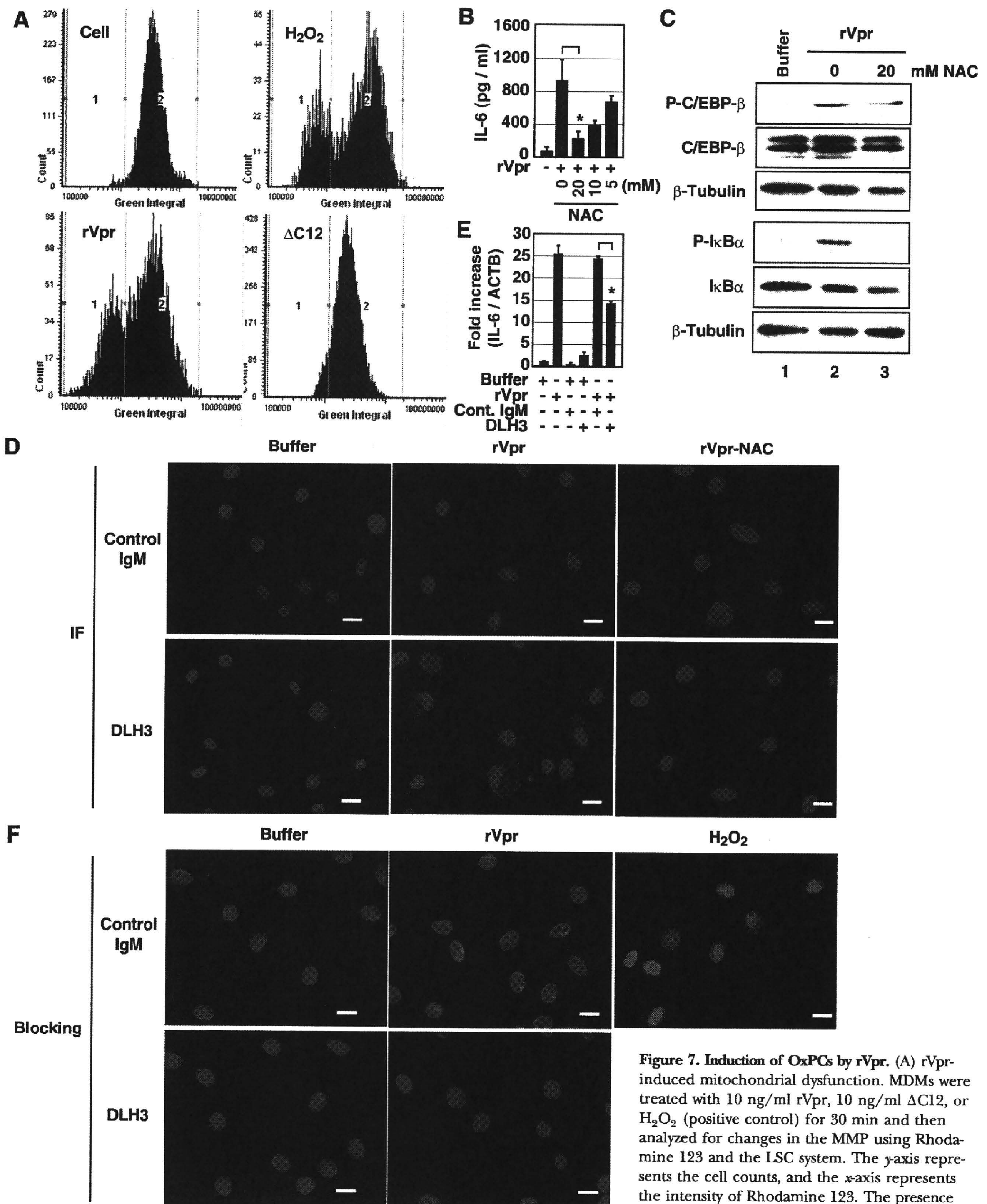

Figure 7. Induction of OxPCs by rVpr. (A) rVpr-induced mitochondrial dysfunction. MDMs were treated with 10 ng/ml rVpr, 10 ng/ml ΔC12, or H_2O_2 (positive control) for 30 min and then analyzed for changes in the MMP using Rhodamine 123 and the LSC system. The *y*-axis represents the cell counts, and the *x*-axis represents the intensity of Rhodamine 123. The presence of a peak with reduced fluorescence intensity indicates a decrease in the MMP [17]. (B) NAC inhibited rVpr-induced IL-6 production. MDMs were treated with NAC at 5, 10, or 20 mM in the presence of 10 ng/ml rVpr for 2 days. (C) The rVpr-induced phosphorylation of C/EBP-β and IκB-α was inhibited by NAC. MDMs were treated with 10 ng/ml rVpr in the presence (lane 3) or

(*continued on next page*)

As Vpr was reported to activate the NF-κB and MAPK pathways [36], we next examined the functional linkage of IκB-α phosphorylation with rVpr-induced IL-6 production. First, we observed that SS, a known inhibitor of IKK [37], blocked rVpr-induced IL-6 production from PBMCs (Fig. 2B) and MDMs (Fig. 2C). Consistent with this, we found that IκB-α was phosphorylated by rVpr (Fig. 2D, lane 3). To examine further the importance of IκB-α phosphorylation in *IL-6* transcription, we prepared a Vpr mutant lacking the ΔC12 (Fig. 2E). Previously, we found that the C-terminal region of Vpr was important for its function, and among several mutants lacking various lengths of the C-terminal region, ΔC12 was almost totally defective in terms of its biological activity compared with wild-type Vpr [38]. Compared with rVpr, ΔC12 showed significantly less ability to induce IL-6 production ($P<0.05$: Fig. 2, F and G). This mutant, however, induced the phosphorylation of IκB-α to a level comparable with that of rVpr (Fig. 2D, lane 4). These data indicate that rVpr-induced IL-6 production depends on the NF-κB pathway but that additional cellular factors are also required.

C/EBP-β is a pivotal factor in rVpr-induced IL-6 production

We next studied the involvement of AP-1 and C/EBP-β in rVpr-induced IL-6 production. WB analysis revealed that AP-1 was activated by rVpr and ΔC12 (**Fig. 3A**, middle column, lane 3), whereas C/EBP-β was activated selectively by rVpr (Fig. 3A, top column, lane 2). Additionally, SB202190, a specific inhibitor of p38, attenuated rVpr-induced IL-6 production ($P<0.05$; Fig. 3B, indicated by *) with concomitant inhibition of rVpr-induced C/EBP-β phosphorylation (Fig. 3C, lane 3). Moreover, SB202190 blocked viral production in U1 cells cocultured with PBMCs and rVpr ($P<0.05$; Fig. 3D). These observations indicate that C/EBP-β is a pivotal factor in rVpr-induced IL-6 production and viral production. To demonstrate this, we tested the effects of C/EBP-β siRNA on rVpr activity. Consistent with the data obtained using SB202190 (Fig. 3B), the knockdown of endogenous C/EBP-β expression abrogated rVpr-induced IL-6 production ($P<0.05$; Fig. 3, E and F).

To highlight further the importance of C/EBP-β in rVpr-induced IL-6 production, we conducted complementation experiments. As shown in **Figure 4A**, U1 (lane 3), its parental cell line U937 (lane 2), and THP-1 (lane 5), another human monocytic leukemia cell line, expressed low levels of endogenous C/EBP-β compared with MDMs (lanes 1 and 4). These three cell lines did not respond to rVpr by producing IL-6 (data not shown); however, the forced expression of exogenous *C/EBP-β* cDNA in leukemic cells (Fig. 4A, lane 7) made the cells competent for rVpr ($P<0.05$; Fig. 4B)- and LPS-induced IL-6 production ($P<0.05$; Fig. 4C). These results indicate that C/EBP-β is a pivotal factor in rVpr activity. Intriguingly, ΔC12 induced less IL-6, even under these conditions ($P<0.05$; Fig. 4B).

rVpr-induced IL-6 production depends on MyD88 and TLR4

To explore the involvement of the TLR signaling cascade in the modulation of NF-κB and MAPK by rVpr, we first examined the effects of the down-regulation of endogenous MyD88 on IL-6 production. The introduction of MyD88 siRNA into MDMs efficiently repressed endogenous MyD88 expression and responsiveness to LPS (**Fig. 5A** and Supplemental Fig. 6). Next, we examined the responsiveness to rVpr and found that rVpr-induced IL-6 production was attenuated significantly by the siRNA ($P<0.05$; Fig. 5B). We further characterized the phosphorylation status of several cellular proteins after the down-regulation of endogenous MyD88. Introduction of the siRNA reduced the rVpr-induced phosphorylation of IκB-α, C/EBP-β, and c-Jun significantly (Fig. 5C, compare lanes 3 and 6). Interestingly, the total amount of IκB-α was increased by rVpr (Fig. 5C, second column from the top, lane 6) but not by LPS. We obtained the same results in three independent experiments, suggesting that Vpr affects the processing of IκB-α, as postulated by Ayyavoo et al. [15].

Next, we examined the involvement of TLR molecules in this process. Among the TLR members tested, TLR2 and TLR4 were of primary interest, as these molecules were reported to interact with viral proteins [22, 23]. As an initial trial, we tested the effects of neutralizing antibodies against TLR2 and TLR4 and found that an anti-TLR4 antibody attenuated IL-6 production by rVpr ($P<0.05$; **Table** 1). We next analyzed the effects of TLR4 siRNA on rVpr-induced IL-6 production. The down-regulation of endogenous TLR4 was confirmed by RT-PCR (**Fig. 6A**), immunohistochemical analysis ($P<0.05$; Fig. 6, B and C), and LPS responsiveness testing ($P<0.05$; Fig. 6D). Two independent experiments were done using MDMs prepared from different healthy donors; both trials revealed that TLR4 siRNA attenuated rVpr-induced IL-6 production significantly ($P<0.05$; Fig. 6E).

rVpr-induced OxPC formation stimulates IL-6 production

Based on our results showing that rVpr-induced IL-6 production depended on TLR4/MyD88, we expected that rVpr would bind TLR4 directly. To demonstrate this, we examined the molecular interaction between rTLR4 proteins and rVpr using the Biacore system but obtained no positive results (data not shown). As another possibility, we hypothesized that TLR4/

absence (lanes 1 and 2) of 20 mM NAC. For the analysis of IκB-α, cells were treated with 150 μM ALLN. (D) Induction of OxPCs by rVpr. MDMs were treated with 10 ng/ml rVpr in the presence or absence of 20 mM NAC. Thereafter, the cells were fixed and stained using DLH3. Red, OxPCs; blue, nucleus; original scale bars, 10 μm. IF, immunofluorescence. (E) rVpr-induced IL-6 production was blocked by inhibiting the induction of OxPCs. MDMs were treated with 10 ng/ml rVpr in the presence or absence of 20 nM DLH3. mRNA samples were then collected, and the level of IL-6 expression was analyzed by RT-qPCR. ACTB was included as a loading control. (F) The rVpr-induced phosphorylation of C/EBP-β was blocked by inhibiting the induction of OxPCs. MDMs were treated with rVpr in the presence or absence of 20 nM DLH3. Thereafter, the cells were fixed and stained using α-phosphorylated C/EBP-β. Red, phosphorylated C/EBP-β; blue, nuclear DNA; original scale bars, 10 μm. The data were obtained from three independent experiments. The values are expressed as the mean ± SD. *, $P<0.05$.

MyD88-dependent pathways were modulated by rVpr-induced oxidative stress and OxPC.

To investigate the involvement of OxPC in triggering rVpr-induced cellular responses, we first sought to determine whether IL-6 production was coupled with rVpr-induced oxidative stress by testing for changes in the MMP, an indicator of oxidative stress [17]. As observed in MDMs treated with H_2O_2 as a positive control (**Fig. 7A**, upper right panel), we found that rVpr generated a peak that was shifted to the left on the *x*-axis, indicating a decrease in the MMP (Fig. 7A, lower left panel; peak 1). In contrast, ΔC12 did not induce a shift (Fig. 7A, lower right panel; only peak 2 is observed). To show the importance of ROS in rVpr-induced IL-6 production, we next examined the effects of NAC on rVpr-induced cellular responses (Fig. 7B). As shown in Figure 7C, NAC decreased the rVpr-induced phosphorylation of C/EBP-β and IκB-α (Fig. 7C, compare lanes 2 and 3). We then examined the formation of OxPC following the addition of rVpr to MDMs. Interestingly, the mAb DLH3 [33, 34] detected OxPC, formed after treatment of rVpr (Fig. 7D, lower middle panel). The rVpr-induced formation of OxPC was blocked by the addition of NAC (Fig. 7D, lower right panel). Moreover, pretreatment of the MDMs with DLH3 attenuated rVpr-induced IL-6 production ($P<0.05$; Fig. 7E) and the phosphorylation of C/EBP-β (Fig. 7F, compare the upper middle and lower middle panels). These data suggest that OxPC, generated by rVpr-induced oxidative stress, modulate TLR4/MyD88 signaling and induce IL-6 production via the phosphorylation of C/EBP-β.

DISCUSSION

In this study, we found that rVpr activated the TLR4/MyD88-dependent cellular cascade in human MDMs, leading to the production of IL-6. rVpr-induced IL-6 production depended on the activation of NF-κB and MAPK signaling and the phosphorylation of C/EBP-β. Moreover, we found that rVpr-induced oxidative stress caused the formation of OxPC, and pretreatment with DLH3, a mAb against OxPC, blocked rVpr-induced IL-6 production and the phosphorylation of C/EBP-β. These data indicate that IL-6 production is initiated by the formation of OxPC generated by the effects of rVpr-induced oxidative stress. Varin et al. [36] reported that a synthetic Vpr peptide activated AP-1, JNK, and NF-κB signaling, resulting in increased viral replication; however, the molecular mechanism involved remains to be clarified. Our current work provides an explanation for the activation of these cellular responses by Vpr-induced oxidative stress. We hypothesize that OxPC, as the most upstream event, extracellularly activates TLR4-mediated signaling. This notion is supported by previous data reported by Imai et al. [27] that TLR4 signaling is activated by acid challenge or treatment with H5N1 in mouse lungs via OxPC. They detected OxPC generated in bronchoalveolar lavage in affected mouse lungs and showed that its activity was blocked by a mAb against OxPC. However, our blocking experiments with DLH3 cannot exclude the possibility that the formation of OxPC and activation of TLR4-mediated cellular signals are required independently for Vpr-induced IL-6 production. Here, we showed that OxPC-triggered cellular signals were dependent on MyD88, whereas those induced by SARS-CoV were shown to be MyD88-independent [27]. These discrepant observations may have been a result of differences in the experimental conditions applied; we used human monocytes/macrophages, whereas Imai et al. [27] used rodent cells.

ROS are likely generated as a result of the mitochondrial dysfunction caused by Vpr. With regard to the mechanism of mitochondrial dysfunction, it has been shown that Vpr binds ANT, a member of the permeability transition pore complex [39], and disrupts the MMP [17]. The domain of Vpr involved in mitochondrial toxicity has been identified as the region encompassing aa 72–83, and arginine residues at positions 73, 77, and 80 (R73, R77, and R80) were defined as being critical for the interaction with ANT [39]. We showed previously that a mutant rVpr protein with alanines in place of the three arginines lacked the ability to impair the MMP and neurite outgrowth [17]. Additionally, a mutant virus carrying Vpr, altered at R77, was detected in patients who were HIV-1-positive and diagnosed as long-term nonprogressors [40], suggesting that the Vpr-induced killing activity of T cells might be linked directly to the clinical outcome of these individuals.

Our finding that exogenously added rVpr reactivated viral reproduction in latently infected cells will broaden our understanding of the pathophysiology in HIV-1-positive patients. HAART dramatically improves the prognosis of HIV-1-positive patients; unfortunately, however, it was concluded that complete eradication of the virus by the chemotherapeutics currently available would take more than 60 years [41]. Such observations underscore the importance of preventing new episodes of viral infection or restraining viral production from latent reservoirs. Vpr has been postulated to function as a necessary factor in the primary infection of resting macrophages and to enhance viral reproduction following latency. As reported previously [13], Vpr is present in the plasma of HIV-1-positive patients, and we ascertained that rVpr added exogenously at the nM level, which is comparable with the level observed in patient plasma, reproducibly reactivated viral production from latency via IL-6 production. Although autoantibodies to Vpr [42] might neutralize its activity, we suggest the importance of monitoring patient plasma Vpr levels in the context of the clinical course. Our results provide a rationale for the development of novel anti-AIDS therapeutics against Vpr.

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ORIGINAL ARTICLE

Whole brain radiation alone produces favourable outcomes for AIDS-related primary central nervous system lymphoma in the HAART era

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Abstract

Primary central nervous system lymphoma (PCNSL) related to acquired immunodeficiency syndrome (AIDS) is a lethal disorder, but the recent application of highly active antiretroviral therapy (HAART) has significantly improved prognosis. This retrospective cohort study of AIDS-related PCNSL examined the actual clinical outcomes and prognostic variables affecting overall survival (OS) in the HAART era. Twenty-three newly diagnosed AIDS-related PCNSL at 12 regional centre hospitals for HIV/AIDS in Japan between 2002 and 2008 were consecutively enrolled. The estimated 3-yr OS rate of the entire cohort was 64% (95%CI, 41.0–80.3%). Whole brain radiation therapy (WBRT) had an independent positive impact on survival (WBRT ≥30 Gy vs. others, $P = 0.02$). Nine of 10 patients with a good performance status (PS) (0–2) remained alive with complete response, whereas 10 (77%) of 13 of those with a poor PS (3–4) died mostly after a short period. The estimated 3-yr OS rate of the groups with a good and poor PS was 100% and 38% (95%CI, 14–63%), respectively ($P = 0.01$). Leukoencephalopathy (grade ≥ 2) developed in 21% of those that survived more than 12 months after radiation. The patients receiving a curative intent radiation dose (≥30 Gy) of WBRT achieved prolonged survival while maintaining a good quality of life in the HAART era, especially among patients with a favourable PS.

Key words acquired immunodeficiency syndrome; primary central nervous system lymphoma; highly active antiretroviral therapy; whole brain radiation; leukoencephalopathy

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Primary central nervous system lymphoma (PCNSL) is one of several acquired immunodeficiency syndrome (AIDS)-defining illnesses (ADI), and it is the second most frequent cerebral mass lesion after toxoplasmosis among those infected with the human immunodeficiency virus (HIV) (1). This type of lymph oma typically arises at the severely immunocompromised late stage of HIV infection, and CD4+ cell counts at diagnosis are

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$<20/\mu L$ in most patients (2, 3). The pathological diagnosis is usually diffuse large B cell lymphoma (4, 5). Although Epstein–Barr virus (EBV) is generally absent from PCSNL in immunocompetent patients, about 80–100% of AIDS-related PCNSL is associated with EBV in lymphoma lesions (6). Pathogenetic roles of EBV infection in AIDS-related PCNSL have been suggested. The incidence of PCSNL has significantly decreased since highly active antiretroviral therapy (HAART) was introduced (7), as have all other types of EBV-positive AIDS-related lymphomas (8). Before the introduction of HAART, the prognosis of AIDS-related PCNSL was dismal and median survival was typically <3 months (9–13). After HAART became available, the clinical outcome of AIDS-related PCNSL radically improved (14–19). However, a standard management procedure for these patients remains to be established. We performed a nationwide retrospective survey to elucidate the actual clinical outcome and to identify the significant prognostic variables of AIDS-related PCNSL in the HAART era, in addition to determining the quality of life of long-term survivors of whole brain radiation.

Patients and methods

This retrospective cohort study examined the clinical outcomes of patients diagnosed with AIDS-related PCNSL (in the HAART era) who visited the 12 regional hospitals for HIV/AIDS in Japan during the period January 2002–December 2008. HAART was defined as two kinds of nucleoside reverse transcriptase inhibitor combined with protease inhibitor or non-nucleoside reverse transcriptase inhibitor. HAART was introduced in 1997 in Japan. This study received approval from the responsible ethics committee.

Patients

The patients included in this study were newly diagnosed with AIDS-related PCNSL during the study period. The pathological diagnosis of each institution was accepted. Those with disseminated lymphoma lesions other than CNS were excluded, whereas those diagnosed with possible AIDS-related PCNSL according to some clinical-based modalities were included. All patients who satisfied the above-mentioned criteria were serially enrolled. Data from all patients registered in this study were statistically analysed.

Clinical characteristics of the patients

Data regarding age, Eastern Cooperative Oncology Group (ECOG) performance status (PS) at diagnosis, number of CD4+ cells at diagnosis, HIV viral load at diagnosis, prior AIDS, concurrent opportunistic diseases, presence of severe neurological symptoms at diagnosis and prior HAART were analysed. Diagnostic modalities and the primary therapy of all enrolled patients were also determined and analysed.

A complete response (CR) to treatment was defined as the disappearance of all clinical evidence of disease at the completion of first induction therapy. The presence of residual disease but with ≥50% decrease in the sum of the products of the greatest diameter was defined as a partial response (PR). Intra-ocular lesions were not assessed in any of the patients. Overall survival (OS) was defined as the interval from diagnosis to death from any cause. Grades of leukoencephalopathy were evaluated based on each institutional decision according to CTCAE v3.0 (20).

Statistical analysis

The primary endpoint of this study was the identification of factors that significantly impacted OS. Both multivariate and univariate Cox regression analyses were performed to assess the effects of treatment and the various baseline prognostic factors on OS. All P values are two-tailed. OS was assessed using the Kaplan–Meier method. Groups divided by clinical variables were compared using the log-rank test. Data were statistically analysed using STATA 10.0 (STATACORP LP, College Station, TX, USA).

Results

Patients' background

Table 1 shows the characteristics of the 23 registered patients with AIDS-related PCNSLs. The median age was 41 (21–60), and male gender accounted for 96% of the patients. Eleven patients developed PCNSL as ADI, and 12 patients were diagnosed with AIDS before the development of PCNSL. Radiological imaging examinations were carried out in all 23 patients. Eleven were diagnosed with PCNSL based on both imaging features and the presence of EBV DNA in cerebrospinal fluid by PCR without a brain biopsy, while three were diagnosed by radiological MRI and SPECT imaging, and the favourable response of brain tumour by radiation therapy. One patient was diagnosed at autopsy. PCR tests of EBV genome in cerebrospinal fluid were performed in 20 patients, and 16 patients out of them showed positivity (80%, 16/20). Seven (30%) were treated with HAART at diagnosis; and finally, HAART was administered to 91% of the patients. Concurrent opportunistic diseases were identified in 15 (65%). Twelve patients had other ADIs before the diagnosis of PCSNL. The median count

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Table 1 Characteristics of patients with AIDS-related PCNSL (n = 23)

Gender	*n* (%)
Male	22 (96%)
Female	1 (4%)
Age(years)	
Median	41
Range	21–60
AIDS diagnosed before PCNSL, n (%)	12 (52 %)
Pneumocystis jiroveci pneumonia	6
Cytomegalovirus infection	2
Candidiasis	3
Cryptosporidiosis	1
HAART therapy before PCNSL, *n* (%)	7 (30%)
Opportunistic diseases at diagnosis of PCNSL, *n* (%)	15 (65%)
CD4+ cell count at PCNSL diagnosis (cells/mL)	
Median	22
Range	1–657
HIV viral load at diagnosis of PCSNL (copy/mL)	
Median	77000
Range	0–1.23 × 10^7
PS at diagnosis of PCNSL, n (%)	
0	2 (7%)
1	4 (17%)
2	4 (17%)
3	5 (22%)
4	8 (35%)
Ataxia and/or cognitive disturbance (grade ≥ 3) at PCNSL diagnosis, *n* (%)	12 (52%)
Diagnostic modality, n (%)	
Biopsy	8 (35%)
Imaging only	3 (13%)
Autopsy	1 (4%)
Positive for EBV genome in CSF by PCR	11 (48%)
PCR test of EBV genome in CSF	(n = 20)
Positive	16/20
Negative	4/20
HAART after diagnosis of PCNSL, *n* (%)	21 (91%)

HAART, highly active antiretroviral therapy; PCNSL, Primary central nervous system lymphoma.
Neurological symptoms graded according to CTCAE v3.0.

of CD4+ cells at PCNSL diagnosis was 22/μL (1–657), and 13 (57%) of 23 patients had a poor PS at diagnosis (3–4). Twelve patients (52%) had severe neurological symptoms defined as ataxia or cognitive disturbance grade ≥ 3 according to CTCAE v3.0 at the time of PCNSL diagnosis.

Treatment and initial response

Twenty-one patients were treated by radiotherapy alone, and only one received combined modality treatment (high-dose methotrexate and cytoxan followed by whole brain radiation (WBRT)). One patient received only best supportive care (BSC). Thirteen patients received a curative intent radiation dose (≥30 Gy) of WBRT. The

Table 2 Initial treatment modality and early clinical response

Treatment modality	*n* (%)	CR/PR, *n* (%)
Whole brain radiation ± local boost (≥30 Gy)	13 (57%)	10 (77%)
Whole brain radiation (<30 Gy)	5 (22%)	1 (20%)
Local brain radiation	3 (13%)	3 (100%)
Combined modality therapy	1 (4%)	1 (100%)
Best supportive care	1 (4%)	0 (0%)
Total	23	15 (65%)

Combined modality therapy: high-dose methotrexate and high-dose cytoxan followed by WBRT.

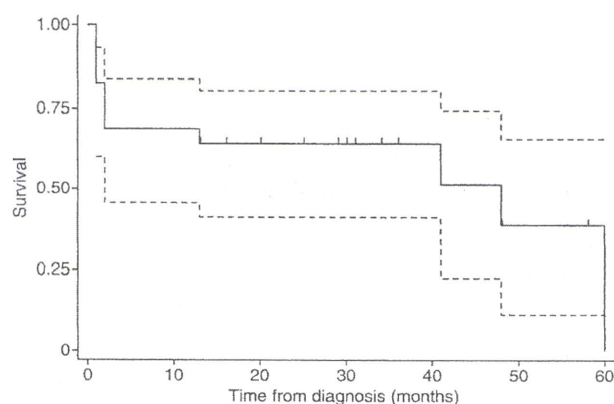

Figure 1 Overall survival curves (All patients with Primary central nervous system lymphoma). Kaplan–Meier estimate with 95% CI (dashed line). Marks indicated censored observation. The total number of censored cases was 12.

overall response rate to all of these strategies including BSC was 65%, while the response rate to a curative intent WBRT was 77% (Table 2).

Clinical variables affecting OS

The estimated 3-yr OS rate of all patients was 64% (95% CI, 41.0–80.3%) with a 20-month median follow-up (Fig. 1).

Significant clinical variables that affected OS were distinguished using univariate and multivariate analyses. Univariate analysis showed that better PS (ECOG) at diagnosis (0–2 vs. 3–4) and receiving curative intent radiation dose (≥30 Gy) of WBRT (WBRT ≥ 30 Gy vs. others) were significant positive survival predictors (P = 0.01 and < 0.01, respectively), and younger age (< 40 yr vs. ≥40) also tended to affect positively on OS but did not reach statistical significance (P = 0.12) (Table 3). Multivariate analysis of these three variables revealed that receipt of WBRT (≥30Gy) had an independent positive impact on OS (P = 0.02) (Table 4). Favourable PS (ECOG) was second strong predictor to

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Table 3 Factors affecting OS (univariate analysis)

Clinical variables	No. of patients	Median survival (Month)(95% CI)	*P* value*
Age (yr)			
<40	9	41 (1.7–80.3)	0.12
≥40	14	2 (2–34.1)	
PS (ECOG)			
0–2	10	48 (N/A)	0.01
3–4	13	2 (0–12.6)	
CD4 (cells/mL)			
<50	18	60 (N/A)	0.77
≥50	5	41 (0–97.7)	
Prior AIDS			
(–)	11	48 (0–114.1)	0.69
(+)	12	41(0–93.2)	
Prior HAART			
(–)	16	41 (0.6–81.4)	0.52
(+)	7	48(12.2–83.9)	
HIV viral load (copy/mL)			
$\leq 1 \times 10^5$	13	13 (N/A)	0.07
$>1 \times 10^5$	10	60 (N/A)	
Severe neurological symptoms at PCNSL onset			
(–)	12	NR	0.12
(+)	11	41 (12.3–69.7)	
Opportunistic disease			
(–)	8	48 (33.8–62.2)	0.34
(+)	15	NR	
Therapy			
WBRT (≥30 Gy)	13	60 (N/A)	<0.01
Other	10	2 (0.7–3.3)	
Response rate			
SD/PD	8	2 (N/A)	0.14
CR/PR	15	48(31.7–64.3)	

*Log-rank test.
CR, complete response; ECOG, Eastern Cooperative Oncology Group; HAART, highly active antiretroviral therapy; N/A, not applicable; NR, not reached; OS, overall survial; PCNSL, Primary central nervous system lymphoma; PR, partial response; WBRT, whole brain radiation therapy.

Table 4 Factors affecting OS (multivariate analysis)

Clinical variables	Hazard Ratio (95% CI)	*P* value
Age		
<40	1	0.09
≥40	5.27 (0.76–36.1)	
PS		
0–2	1	0.06
3–4	9.24 (0.86–96.43)	
Therapy		
WBRT (≥30Gy)	1	0.02
Other	8.10 (1.35–48.43)	

OS, overall survial; PS, performance status; WBRT, whole brain radiation therapy.

better OS with highest hazard ratio but was not statistically significant ($P = 0.06$).

Nine of ten patients with a good PS (0–2) remained alive with CR (all received curative intent WBRT), nevertheless 10 (77%) of 13 of those with a poor PS (3–4) died mostly within 2 months (7/10; 70%). The estimated 3-yr OS rate of each group was 100% and 38% (95% CI, 14–63%), respectively ($P = 0.01$, log-rank test) (Fig. 2A).

The 3-yr OS rates for 13 patients who received WBRT (≥30Gy) estimated from Kaplan–Meier survival curves and in the group that received a different type of treatment were 92% (95% CI, 57–99%) and 24% (95% CI, 4–58%), respectively ($P < 0.01$, log-rank test) (Fig. 2B).

Leukoencephalopathy and PS in survivors

Leukoencephalopathy is a late-onset, serious adverse event associated with radiation therapy to the brain

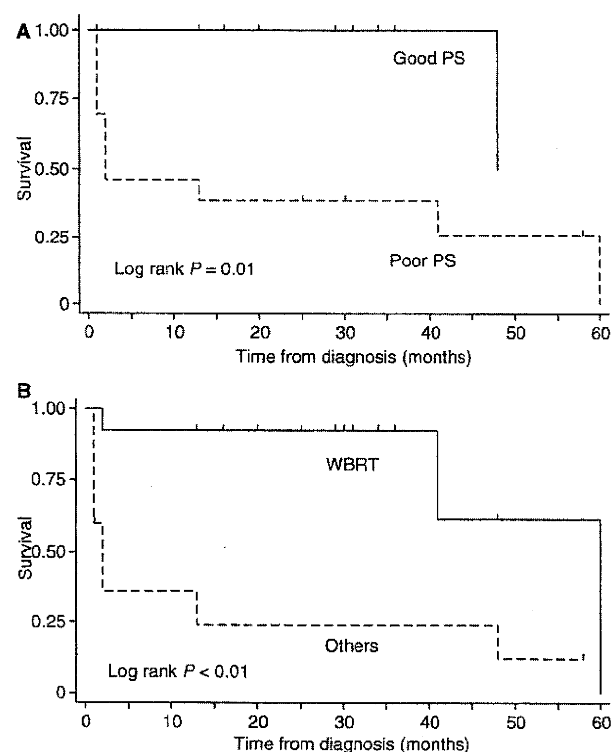

Figure 2 Overall survival curves. (A) Survival according to performance status (PS) at Primary central nervous system lymphoma(PCNSL) diagnosis. Solid line, patients with PS 0–2 (good PS); dashed line, patients with PS 3–4 (poor PS). Marks indicated censored observation. The number of censored cases was nine in good PS group and three in poor PS group. (B) Survival according to initial therapy for PCNSL. Solid line, patients receiving WBRT (≥30 Gy); dashed line, patients receiving other therapy. Marks indicated censored observation. The number of censored cases was 10 in WBRT (≥30 Gy) group and two in others' group.

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Table 5 Current status and neurological symptoms of patients who survived ≥12 months

Patient No.	Survival (months)	Neurological symptoms Ataxia/cognitive disturbance	Leukoencephalopathy	PS
1	48	0/2	1	1
2	25	4/3	3	4
3	30	0/0	0	0
4	31	3/0	0	1
5	34	2/1	0	1
6	13	0/0	0	0
7	58	1/0	1	1
8	20	0/1	0	1
9	16	0/0	0	0
10	29	0/0	0	0
11	36	0/0	0	0

PS, performance status.
Neurological symptoms and leukoencephalopathy graded according CTCAE v3.0.

(21–23). We analysed the incidence and grade of radiation-related leukoencephalopathy, which was assessed among the patients who survived for ≥12 months after initial radiation therapy. Leukoencephalopathy was graded according to CTCAE v3.0. Twelve patients survived for ≥12 months after WBRT (≥30 Gy), and two patients survived for ≥12 months after local brain radiation. Among these fourteen patients, five (36%) were diagnosed with leukoencephalopathy by CT or MRI imaging, and three of them had leukoencephalopathy grade ≥ 2 (median follow-up, 30 months; range, 13–58 months). No signs of leukoencephalopathy have developed in eight of the 12 survivors who received WBRT (≥30 Gy).

We also analysed the current neurological symptoms and PS of 11 living patients. The PS of all patients except for one with severe neurological symptoms was ≤1 (Table 5).

Discussion

AIDS-related PCNSL was a highly lethal ADI in the pre-HAART era, with survival being generally quoted as <3 months (9–13). Many studies have indicated improved survival of patients with AIDS-related PCNSL after the introduction of HAART (14–19), but standard management for such patients has not been established.

Our retrospective cohort study of AIDS-related PCNSL in the HAART era showed favourable survival especially in patients with a good PS who underwent WBRT at the dosage of ≥30 Gy designed for curative intent. Univariate analysis showed that significant clinical factors for a favourable OS were a good PS (ECOG 0–2) at diagnosis and the receipt of WBRT (≥30 Gy). Multivariate analysis selected the receipt of WBRT (≥30 Gy) as the statistically significant clinical factor for a favourable OS. Even in the HAART era, low CD4+ cell counts was reported to be a significant poor prognostic factors for AIDS-related systemic non-Hodgkin lymphoma (24). Our data could not show that CD4+ cell count had the prognostic effect in AIDS-related PCNSL in the HAART era. Systemic non-Hodgkin lymphomas were usually treated with systemic chemotherapy, which could impair host immune status, and one of the major causes of death was severe infection during treatment. Thus CD4+ cell count in AIDS-related systemic lymphoma would be more important than in PCNSL treated with brain radiation, which might have minimal damages to host immunity, in the context of control of infectious complications.

Some reports during the HAART era have indicated improved survivals of patients with PCNSL after treatment with curative intent WBRT. However, in each study, all patients with PCNSL were not reported to be actually treated with this modality in the HAART era; the largest study comprised 25 patients (16), but only 10 of the patients described in that study underwent both WBRT (≥30 Gy) and therapy with two or three anti-retroviral agents. All of our 23 patients were diagnosed in the HAART era, 12 were treated with both HAART and the curative intent WBRT, and we followed up the survivors for longer (median: 18 months) than any other studies (14–16). The 3-yr OSs of the entire cohort, the group with a favourable PS, and the group that underwent WBRT were 64%, 100% and 92%, respectively. These data showed that the survival of patients with AIDS-related PCNSL could be favourable if treated with curative WBRT under a relatively good general PS during the HAART era. The reported 3-yr OS of patients with non-AIDS-related PCNSL is 29% when treated only with brain radiation (25) and 50–70% when treated with high-dose MTX-based chemotherapy plus brain radiation (26, 27). Our survival findings were comparable with those of immunocompetent patients and might be superior if PS is favourable at diagnosis.

One major difference between AIDS-related and immunocompetent PCNSL is considered the consistent association with EBV. The presence of EBV in the setting of prolonged immunosuppression might cause B cell activation that result in the development of PCNSL. Anti EBV therapy or HAART with/without ganciclovir and interleukin two have been applied to treat AIDS-related PCNSL, with some good responses (28–30). In the context of these concepts, the role of chemotherapy in AIDS-related PCNSL remains obscure. The adequacy of such therapeutic modalities, as WBRT, a high-dose MTX-based regimen, and com-

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bined therapy should be further analysed in prospective clinical trials.

Our long-term follow-up allowed an analysis of the incidence of leukoencephalopathy, general status and neurological symptoms after therapy was completed. The adverse effects of brain radiation comprise an acute type that can occur even during radiation, an early-delayed type that occurs 2–4 months later, and a late type that manifests about 9–12 months later. Leukoencephalopathy is a late-onset complication that requires long-term follow-up. Our patients were followed up for 13–56 months, which should have allowed most leukoencephalopathy to be recognised. The incidence was 36% (5/14), and severe events (grade ≥ 2) developed in three patients. The PS of all eleven survivors except for one with grade 3 leukoencephalopathy was ≤1. Two patients showed cognitive disturbance of grade ≥ 2, and three showed ataxia of grade ≥ 2. PCNSL itself, even in the remission status, might account for some neurological symptoms. Longer observation might be required to determine the final outcome of late-onset radiation-damage to the brain.

Our findings suggested that patients with AIDS-related PCNSL achieved durable remission after curative intent WBRT, especially those with a good PS during the HAART era. These findings indicate that early diagnosis of this disease before symptoms can affect general status could result in prolonged survival with a favourable outcome. Thus, surveillance of a high-risk population for HIV infection and close follow-up of patients infected with HIV should improve the outcomes of AIDS-related PCNSL.

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症例

急性 Epstein-Barr ウイルス感染症を発症した HIV 感染症の 1 例*

古西 満　米川真輔　宇野健司
善本英一郎　三笠桂一**

はじめに

HIV (human immunodeficiency virus) と EBV (Epstein-Barr virus) との重複感染はしばしばみられる．HIV 感染者で臨床的に問題となる EBV 感染に関連した病態は，潜伏感染していた EBV の再活性化による舌毛状白板症[1]や悪性リンパ腫[2]などである．今回われわれは，EBV 初感染による伝染性単核症を発症した HIV 感染症症例を経験し，興味深い血液検査所見を認めたので，報告する．

症　例

症　例：30 歳，男性．

主　訴：倦怠感．

家族歴：父が脳腫瘍．

既往歴：特記すべきことなし．

現病歴：血友病 A のため使用した非加熱血液製剤で HIV に感染したが，無治療でも病状は安定していた (CD4 陽性細胞数 500/μl 以上，HIV-RNA 量 10^2～10^3コピー/ml 台)．2008 年 4 月中旬に 39℃台の発熱があり，以後倦怠感が持続していた．歯性上顎洞炎のため通院していた当院口腔外科での血液検査で，肝機能障害，異型リンパ球の出現を認めたため，4 月下旬に当科を受診した．

身体所見：身長 180 cm，体重 78 kg，血圧 126/70 mmHg，脈拍 92/min・整，体温 37.1℃．扁桃腫大を認め，両側頸部に 5～10 mm 前後のリンパ節を数個触知した．胸・腹部には異常所見はなかった．顔面の淡い紅潮と体幹部のわずかな丘疹を認めた．

受診時検査所見 (Table 1)：白血球数は増加し，71% が異型リンパ球であった (Fig. 1)．軽度の肝機能障害があり，EBV の初感染を示唆する抗体検査結果を認めた．頸部単純 CT 写真ではリンパ節腫大を認めた (Fig. 2)．

臨床経過 (Fig. 3)：自覚症状，身体所見および血液検査所見は自然経過で改善した．しかしそれまで 10^2～10^3台のコピー数で推移していた HIV-RNA 量が，EBV 感染後一時的に 1.4×10^5コピー/ml に急増し，CD4 陽性細胞数も減少傾向を示した．

考　察

日本人では成人の 90% 以上で EBV 抗体を保有しており，EBV はきわめて普遍的なウイルスの一つである．EBV 初感染の多くは無症候性であるが，思春期以降の感染では伝染性単核症として発症する割合が高くなる．伝染性単核症の臨床症状には，発熱，咽頭・扁桃炎，リンパ節腫脹，肝脾腫，発疹，結膜充血などがある．検査所見では，白血球増加 (リンパ球優位)，異型リンパ球出現，肝機能異常などがみられる．EBV 感染以外にサイトメガロウイルスや HIV の初感染時にも同様の病態を示すことがあるので，鑑別する必要がある．

診断は EBV 関連抗体検査によることが一般的で，急性期に EBNA 抗体陰性で VCA IgG 抗体が陽性であり (初感染パターンと呼ばれる)，加えて急性期に VCM IgM 抗体が陽性で，回復期に VCA

* A Case of Primary Epstein-Barr Virus Infection in an HIV-Positive Patient.

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Table 1. 受診時検査所見

末梢血		CK	32 IU/l	CMV 抗原(C7-HRP)	(−)
WBC	13,100/μl	BUN	7 mg/dl	EBV VCA IgM	40 倍
好中球	12%	Cr	0.80 mg/dl	EBV VCA IgA	20 倍
リンパ球	11%	Na	136 mEq/l	EBV VCA IgG	160 倍
単球	6%	K	4.3 mEq/l	EBV EA-DR IgA	<10 倍
異型リンパ球	71%	Cl	102 mEq/l	EBV EA-DR IgG	10 倍
RBC	$443\times10^4/\mu l$	Glu	106 mg/dl	EBV EBNA	<10 倍
Hb	13.1 g/dl	血清		HTLV-1 抗体	(−)
Ht	38.8%	CRP	0.7 mg/dl	ガラス板法	(−)
Plt	$13.9\times10^4/\mu l$	可溶性 IL-2 レセプター		TP 抗体	(−)
生化学			3,490 U/ml	HBs 抗原	(−)
AST	74 IU/l	感染症		HCV 抗体	(+)
ALT	50 IU/l	CMV IgM	0.54		
LDH	565 IU/l	CMV IgG	<2.0		

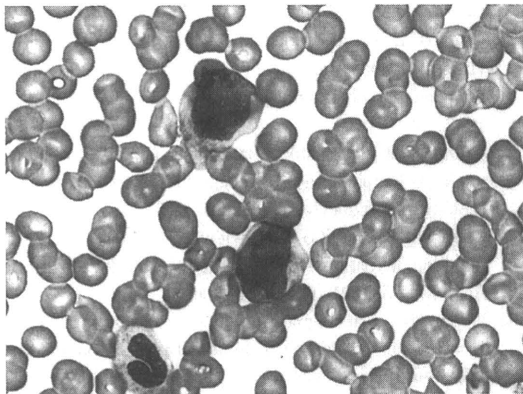


Fig. 1. 末梢血塗抹標本

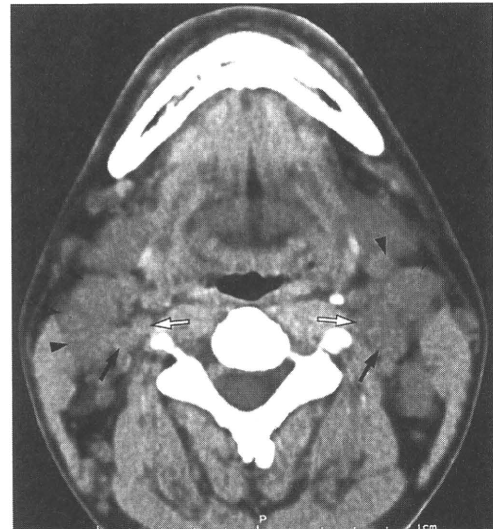
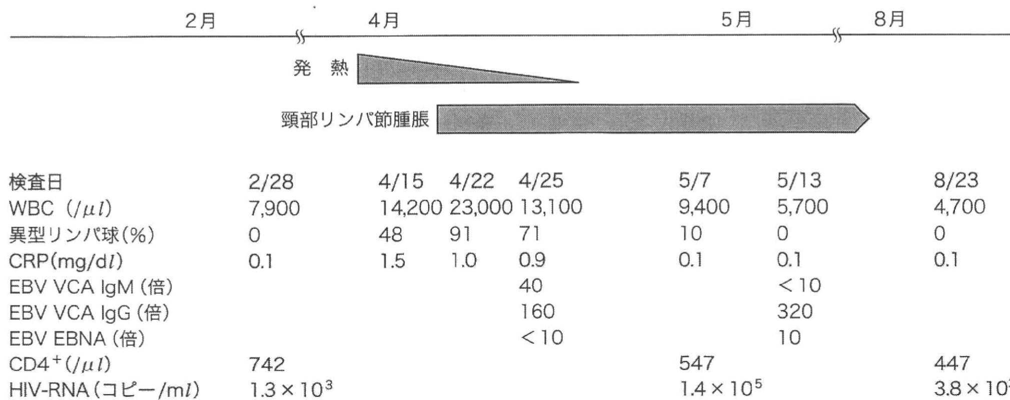


Fig. 2. 頸部単純 CT 画像
黒矢印：内頸静脈，白矢印：総頸動脈，矢頭：腫大リンパ節．

IgM 抗体が陰性化し EBNA 抗体が陽性化すれば診断は確実である．本症例では，臨床症状，検査所見，EBV 関連抗体検査から，EBV 初感染による伝染性単核症と診断することに問題はないと考える．

EBV は唾液を介してヒトの口腔咽頭上皮に感染後，B リンパ球の CD21(C3d レセプター)に EBV の gp350/220 が結合して B リンパ球への感染が成立する[3]．感染した B リンパ球は不死化し B リンパ芽球になるが，CTL(cytotoxic T lymphocyte)による EBV 特異的細胞傷害活性が機能すると B リンパ芽球は体内から排除される．ところが，休止期 B リンパ球内の EBV は CTL による免疫機構から逃れ，潜伏感染の状態となる．HIV 感染症で免疫能が低下すると，EBV の再活性化に伴って不死化した B 細胞を排除することができなくなり，リンパ増殖性疾患や悪性リンパ腫の発症にいたることがある[4]．HIV 感染症の免疫不全に伴う EBV 再活性化による病態に関する報告は多いが，HIV 感染者の EBV 初感染に関する報告は少ない．検索しえた範囲では，HIV 感染児に上気道閉塞をきたした急性 EBV 感染症の報告[5]の



検査日	2/28	4/15	4/22	4/25	5/7	5/13	8/23
WBC (/μl)	7,900	14,200	23,000	13,100	9,400	5,700	4,700
異型リンパ球(%)	0	48	91	71	10	0	0
CRP(mg/dl)	0.1	1.5	1.0	0.9	0.1	0.1	0.1
EBV VCA IgM (倍)				40		<10	
EBV VCA IgG (倍)				160		320	
EBV EBNA (倍)				<10		10	
$CD4^+$(/μl)	742				547		447
HIV-RNA (コピー/ml)	1.3×10^3				1.4×10^5		3.8×10^3

Fig. 3. 臨床経過

みである．

本症例では伝染性単核症の経過は一般的なものと同様であったが，EBV 感染後に血漿中 HIV-RNA 量が一時的に増加しており，興味深い点である．Moriuchi ら[6]は，急性 EBV 感染者で CD4 陽性細胞の CCR5 発現が亢進し，R5-HIV の感染性が増強すると報告している．本症例の HIV が R5-HIV か X4-HIV かは確認できていないが，一時的に血漿中の HIV-RNA 量が増加した現象に関連している可能性がある．また，HIV 感染者での EBV 初感染後の経過もよくわかってないので，本症例の経過を注意深く追っていく必要があると考える．

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感染症診療ゴールデンハンドブック

編集●藤田次郎／喜舎場朝和

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研修医・若手医師を対象に感染症診療の具体的ポイントをコンパクトにまとめ，携帯に便利な新書判で提供．感染症診療の「基本アプローチ」，各感染症の症状，検査，起炎菌，治療，処方をまとめた「各感染症へのアプローチ」，抗菌薬，抗真菌薬，抗ウイルス薬の各分類，適応，投与量，副作用をまとめた「薬剤からのアプローチ」を中心に実践的内容を箇条書きで記す．

研究成果の刊行に関する一覧表
平成22年度 国立病院機構 東京病院 永井英明

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Komiya K, Ariga H, Nagai H, Teramoto S, Kurashima A, Shoji S, Nakajima Y.	Impact of peripheral lymphocyte count on the sensitivity of 2 IFN-gamma release assays, QFT-G and ELISPOT, in patients with pulmonary tuberculosis.	Intern Med	49	1849-1855	2010
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INTERNAL MEDICINE

□ ORIGINAL ARTICLE □

Impact of Peripheral Lymphocyte Count on the Sensitivity of 2 IFN-γ Release Assays, QFT-G and ELISPOT, in Patients with Pulmonary Tuberculosis

Kosaku Komiya[1,2], Haruyuki Ariga[1], Hideaki Nagai[1], Shinji Teramoto[1], Atsuyuki Kurashima[1], Syunsuke Shoji[1] and Yutsuki Nakajima[1]

Abstract

Objective This study evaluated the effect of peripheral lymphocyte count on 2 interferon-gamma release assays [QuantiFERON TB-Gold (QFT-G) and enzyme-linked immunospot (ELISPOT)] and their sensitivity in patients with pulmonary tuberculosis, including HIV-negative immunocompromised patients.

Patients and Methods Two hundred thirty patients with microbiologically confirmed active pulmonary tuberculosis were subjected to the tests. Lymphocyte counts were analyzed simultaneously.

Results Overall sensitivity was 74% (159/215; 95% CI, 68-80%) for QFT-G and 92% (198/215; 89-96%) for ELISPOT ($p<0.0001$). In patients with peripheral lymphocyte counts of ≥1000/μL, sensitivity was high for both QFT-G (88%, 111/126; 82-94%) and ELISPOT (97%, 122/126; 94-100%). However, the sensitivity decreased significantly with decreasing peripheral lymphocyte count for both QFT-G (test for trend $p<0.0001$) and ELISPOT (test for trend $p=0.007$). When lymphocyte counts were <500/μL, the sensitivity was 81% (25/31; 66-96%) for ELISPOT, but only 39% (12/31; 21-57%) for QFT-G.

Conclusion Both QFT-G and ELISPOT are sensitive methods for detecting active pulmonary tuberculosis, but their sensitivity partly depends on peripheral lymphocyte counts. At low lymphocyte count conditions, ELISPOT is superior to QFT-G for detecting tuberculosis, irrespective of age, gender, and nutrition.

Key words: IFN-γ release assay, lymphocyte count, pulmonary tuberculosis

(Inter Med 49: 1849-1855, 2010)
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Introduction

Until recently, the detection of latent *Mycobacterium tuberculosis* infection (LTBI) was only based on tuberculin skin testing (TST). With the advent of highly specific interferon-gamma (IFN-γ) release assays (IGRAs), however, alternatives are now available. False-positive results with TST occur because of antigenic cross-reactivity of the purified protein derivative with non-*M. tuberculosis* infections, including those caused by Bacille Calmette-Guerin (BCG) vaccination. In contrast, IGRAs are not affected by BCG vaccination and most non-*M. tuberculosis* infections, and are at least as sensitive as TST for detecting active tuberculosis (1, 2). Therefore, the Centre for Disease Control and Prevention (CDC) guideline recommends that IGRAs are more useful than TST for detection of LTBI (3, 4).

Although the overall sensitivity of IGRAs such as QuantiFERON TB Gold (QFT-G) and enzyme-linked immunospot (ELISPOT) were reported to be very high, these were evaluated only in studies that excluded distinctly immunocompromised patients (5-20). However, immunocompromised hosts, including elderly and patients receiving immunosuppressive agents, have a greater risk of tuberculosis than usual hosts. Some studies on immunocompromised hosts have reported that IGRAs are more useful than TST, but the sensitivity of IGRAs was diminished in those patients (1, 6-9). It has also been reported that CD4 lympho-

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cyte counts affect the positive and intermediate responses of QFT-G (8, 10-15).

Therefore, this study was conducted to quantify the effect of peripheral blood lymphocyte count on QFT-G and ELISPOT and to evaluate their sensitivity in patients with pulmonary tuberculosis, including HIV-negative immunocompromised patients.

Methods

Setting, patient recruitment, and eligibility

In total, 602 patients who were clinically suspected of pulmonary tuberculosis infection were tested with QFT-G and ELISPOT simultaneously, between January 2008 and June 2009 at the Department of Pulmonary Medicine, Tokyo National Hospital, National Health Organization (Tokyo, Japan). Patients provided written and informed consent. Two hundred and fifteen HIV-negative patients with *M. tuberculosis* infection confirmed by positive culture and/or PCR for *M. tuberculosis* from sputum or bronchoalveolar lavage were recruited prospectively. Patients were tested before or within 14 days of initiation of chemotherapy. Information on immunosuppressive therapy, malignancies, and bedridden status was collected from each patient at the time of enrollment. Laboratory findings [peripheral blood cell count, lymphocyte count, serum albumin (Alb), C-reactive protein (CRP), and haemoglobin A1c (HbA1c)], sputum smear status, and radiological findings were obtained at the same time.

QFT-G and ELISPOT were performed by the following procedures. Furthermore, the sensitivity of each assay was calculated and the results were analysed according to peripheral lymphocyte counts. We compared the mean peripheral lymphocyte counts between groups with positive, negative, and indeterminate results of the 2 IGRAs.

Furthermore, univariate and multivariate analyses were performed with respect to the significant contributing factors for the results of the sensitivity of these assays in detecting pulmonary tuberculosis.

This study was approved by the ethics committee of Tokyo National Hospital, National Health Organization (Tokyo, Japan).

QFT-G test

The QFT-G test was performed according to the recommendations of the manufacturer (Cellestis, Ltd., Carnegie, Victoria, Australia), and the test results were evaluated according to the guidelines of CDC (21).

ELISPOT assays

Peripheral blood mononuclear cells were separated from the heparinized blood sample by density centrifugation using BD Vacutainer® Cell Preparation Tubes (Becton, Dickinson and Company, Franklin Lakes, NJ). We seeded pre-coated IFN-γ ELISPOT plates (BD) with 2.5×10^5 cells per well in AIM-V medium (GIBCO), and then incubated with ESAT-6 (5 g/mL) and CFP-10 (5 g/mL) peptides at 37℃ in 5% carbon dioxide for 16 h. A negative control (no mitogen or antigen) and a positive control (phytohemagglutinin, 5 g/mL) were included. After incubation, the wells were washed and developed with a conjugate against the antibody used and an enzyme substrate. Spot-forming units were counted using a KS ELISPOT system. The results of ELISPOT were also interpreted according to the following criteria: The test result is positive if 1) the negative control has 0-5 spots and (ESAT-6 or CFP-10 spot count) (negative control spot count) 6, and/or 2) if the negative control has 6-10 spots and (ESAT-6 or CFP-10 spot count) 2× (negative control spot count). The test result is negative if the above criteria are not met and the positive control is valid. If the positive control is indeterminate (<20 spots) and both antigens are negative, the test result is regarded as indeterminate.

Statistical analysis

Data were entered using PASW statistics 17.0 for Windows and analyzed. Analyses were 2-sided, confidence intervals (CI) were 95%, and results were considered significant when $p<0.05$. Continuous variables were tested for normality using the Shapiro-Wilk test and compared using Student's t-test distribution. In other cases, the Mann-Whitney test and Kruskal-Wallis test were used. The chi-square test was applied for comparing categorical variables unless 1 of the categories had less than 20 observations, in which case, the Fisher's exact test was applied. A chi-square test and a linear trend test were conducted for testing trends. Odds ratio analysis for risk factors was performed by both univariate and multivariate analyses.

Results

Patient characteristics and IGRA results

Two hundred and thirty patients were examined in the study. Demographic and clinical characteristics of the study patients are summarized in Table 1. Two hundred and thirteen were Japanese in origin, and the others were either Chinese or Filipino. A total of 215 were HIV negative. The primary diseases of the 18 patients who were undergoing immunosuppressive treatment were rheumatoid arthritis (7 patients), idiopathic interstitial pneumonia (4), microscopic polyangitis (2), sarcoidosis (1), autoimmune hepatitis (1), aplastic anaemia (1), Crohn disease (1), and Still disease (1).

Overall sensitivity is shown Table 2. Figure 1 and 2 show the distribution of results of ELISPOT according to the QFT-G result and vice versa, respectively. Among the 3 subgroups of 160 (74%) positive, 49 (23%) negative, and 6 (3%) indeterminate QFT-G result subjects, the median peripheral lymphocyte count was highest in the positive group [median: 1,247; interquartile range (IQR): 860, 1,680], intermediate in the negative group (median: 732; IQR: 475, 1,120), and lowest in the indeterminate group (median: 164; IQR: 141, 630) ($p<0.0001$). On the other hand, among the 2