approximately 2.5 μg/ml at 4 °C over night and subsequently blocked with 1% bovine serum albumin, 0.09% sodium azide and 5% sucrose in PBS at room temperature for 2 h. The plates were incubated with 1:100 diluted sera for 1 h at room temperature, washed and then incubated with 1:14,000 diluted horseradish peroxidase-conjugated goat polyclonal anti-human IgG antibodies (MBL) for 1 h at room temperature. After washing the color development was achieved by substrate solution TMBS-1000 (Moss Inc., Maryland). The substrate solution was incubated for 30 min at room temperature and color development was stopped by adding 0.5 M H_2SO_4. The absorbance was measured at 450 nm by microtiter plate readers (Bio-Rad Laboratories, Hercules, CA; Tosoh Co., Tokyo, Japan). The index value was defined by the following formula: index = (optical density [OD] of tested serum − OD of negative control)/(OD of positive control − OD of negative control) × 100. A positive control and negative control were included in every plate. Positive control for BP230 ELISA was a diluted standard BP serum. The negative control was a diluted standard serum obtained from a normal control. The negative control revealed the non-specific, background noise of the system, which was subtracted from all values of the plate.

2.5. BP180 ELISA

The ELISA of NC16a domain of BP180 was performed as reported previously [24,25].

2.6. Statistical analysis

A receiver-operating-characteristic (ROC) analysis was performed to determine a cut-off value for BP230 ELISA. The sensitivity and specificity were plotted when different scores were used for cut-off value and the one which gave the highest specificity (percentage) and specificity (percentage) was determined to be the cut-off value to be used. Data obtained with 72 BP sera and 109 normal control sera were used for this analysis.

2.7. Assessment of disease activity

Disease activity was arbitrarily assessed on a scale of 1–4. The score 4 was set at the highest activity for each patient during the observation period and relative activity was assessed arbitrarily. In this assessment, the remission stages were shown as score 1. The scoring for the relative disease activity was equal for all the patients.

3. Results

3.1. SDS-PAGE and immunoblotting of the BP230 recombinant proteins

SDS-PAGE gel stained with Coomassie brilliant blue of the purified BP230-N and BP230-C showed a single band with predicted mobility (Fig. 1A). Immunoblotting of anti-glutathione-S-transferase (GST) antibodies showed a strong reactivity with these protein bands (Fig. 1B).

3.2. Analysis of sera obtained from patients and normal controls with BP230 ELISA

To determine a cut-off value we performed a receiver-operating-characteristic analysis with 72 BP sera and 109 normal control sera with BP230 ELISA (Fig. 2). The one, which gave the highest sum of sensitivity (percentage) and specificity (percentage), was defined as the cut-off value to be used. For BP230 ELISA, this was determined to be 9.2.

With the cut-off value 9.1, BP180 ELISA showed that 53 (84.4%) of 64 sera from BP patients in active stage and 113 (64.6%) of 175 sera from BP patients in remission were positive; in total 167 (69.9%) of 239 BP sera were positive. With the cut-off value 9.2, BP230 ELISA showed that 37 (57.8%) of 64 sera from BP patients in active stage and 136 (77.7%) of 175 sera from BP patients in remission were positive; in total 173 (72.4%) of 239 BP sera were positive. When the results of BP180 ELISA and BP230 ELISA were combined, 60 (93.8%) of 64 sera from BP patients in active stage and 172 (98.3%) of 175 sera from BP patients in remission were positive; in total 232 (97.1%) of 239 BP sera were positive (Fig. 3 and Table 1). Thus, the sensitivity of BP180 ELISA, BP230 ELISA and combination of BP180 ELISA and BP230 ELISA were 69.9, 72.4 and 97.1%, respectively.

Concerning the specificity, BP180 ELISA showed that none of the 94 sera from pemphigus patients and 5 (1.5%) of the 336 normal control sera were positive. BP230 ELISA showed that one (1.1%) of the 94 sera from pemphigus patients and none of the 109 normal control sera were positive. When the results of BP180 ELISA and BP230 ELISA were combined, one (1.1%) of the 94 sera from pemphigus patients was positive. Thus, the specificity of BP180 ELISA, BP230 ELISA and combination of BP180 ELISA and BP230 ELISA were 98.8, 99.5 and 98.9%, respectively (Fig. 3 and Table 2).

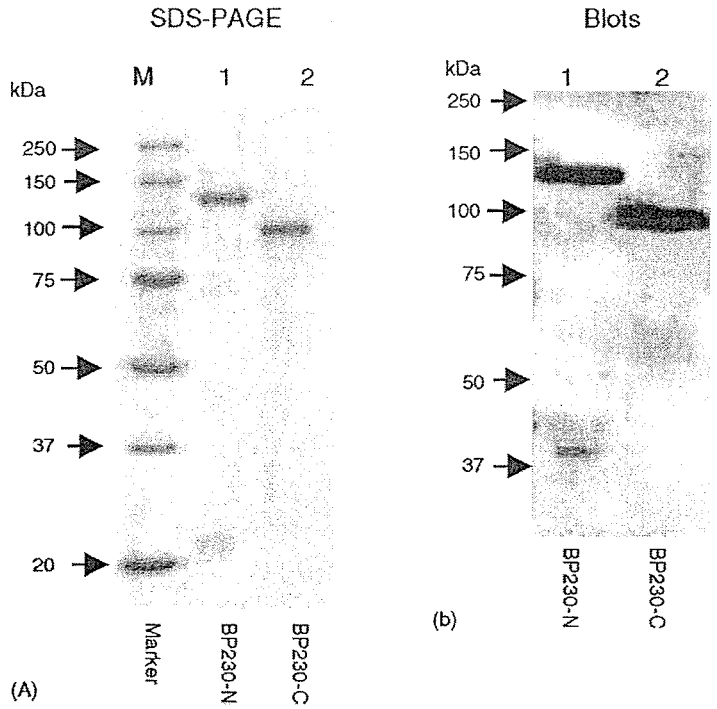

Fig. 1 SDS-PAGE and immunoblotting of the BP230 recombinant proteins. (A) SDS-PAGE gel stained with Coomassie brilliant blue. (B) Immunoblotting using anti-GST antibody.

3.3. The comparison of ELISA scores, indirect immunofluorescence titers and disease activity

We compared ELISA scores, indirect immunofluorescence titers and disease activity over time in 10 BP patients, whose serial sera and clinical records were available. We have selected the 10 BP cases, because we could obtained multiple serum samples from these cases.

In general, the 10 BP cases could be classified into three groups from the results of BP230 ELISA scores in this comparative study (Fig. 4A–J). In the first group (cases 1–5), the BP230 ELISA scores fluctuated in parallel with the disease activity. The BP180 scores also showed a parallel fluctuation with the disease activity in this group. The indirect immunofluorescence titers showed a roughly parallel pattern, although the relationship with the disease activity was not as clear as ELISA scores of BP230 and BP180.

In the second group (cases 6–8), the scores of BP230 ELISA were constantly high and did not decline in parallel with the disease activity. The indirect immunofluorescence titers were also constantly high. Interestingly, in two cases of this group (cases 7 and 8), the BP180 ELISA titers were negative throughout the disease course.

In the third group (cases 9 and 10), the BP230 ELISA scores are constantly negative. In contrast, in this group, the BP180 ELISA titers and the indirect immunofluorescence titers fluctuated in parallel with the disease activity.

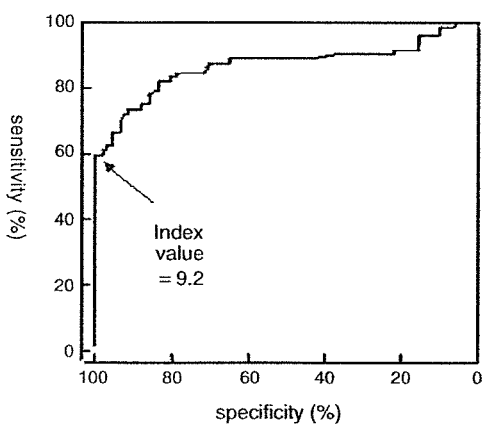

Fig. 2 Receiver-operating-characteristic curve to determine a cut-off value.

4. Discussion

Indirect immunofluorescence using normal human skin sections or monkey esophagus sections as a substrate is a standard serological test for the diagnosis of BP. In addition, immunofluorescence using

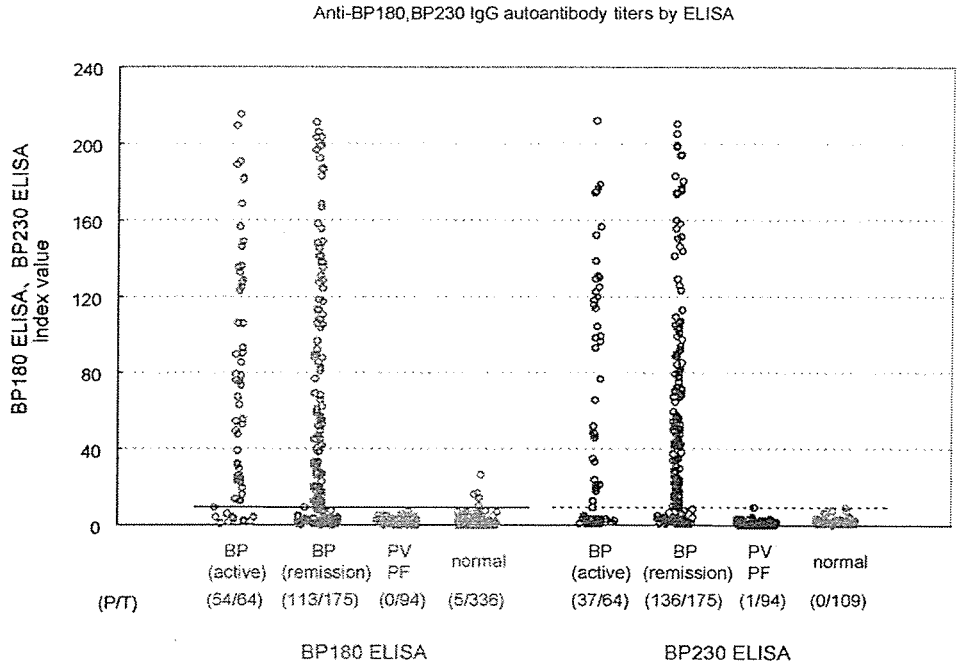

Fig. 3 IgG autoantibody titers by ELISA using recombinant proteins of BP180 (left) and BP230 (right). PV PF: total of pemphigus vulgaris and pemphigus foliaceus sera. Red line indicates the cut-off value for BP180 ELISA (9.1), and dashed line indicates the cut-off value for BP230 ELISA (9.2).

1 M NaCl split skin sections is highly sensitive and more reliable test. However, it is known that the titers of indirect immunofluorescence do not correlated with the disease activity in BP [36–38]. This is probably because indirect immunofluorescence detects not only anti-BP180 antibodies, which were proved to be pathogenic, but also anti-BP230 antibodies, which may not be directly involved in the induction of blister formation.

In the previous study, we developed ELISA using bacterial recombinant protein of BP180 NC16a domain [24]. In the BP180 ELISA, the sensitivity for the BP sera in active stage was 84.4% and that of 20 BP sera in remission was 40%. The sensitivity of the BP180 ELISA was 98.8%. In addition, the index value of the BP180 ELISA was shown to correlate well to the disease activity.

Table 1 Sensitivity of BP230 ELISA and BP180 ELISA for BP sera

	Active stage (n = 64)	Remission (n = 175)	Total (n = 239)
BP230-ELISA	57.8% (37/64)	77.7% (136/175)	72.4% (173/239)
BP180-ELISA	84.4% (54/64)	64.6% (113/175)	69.9% (167/239)
BP230 + BP180	93.8% (60/64)	98.3% (172/175)	97.1% (232/239)

In another previous study, we prepared three bacterial recombinant proteins covering entire human BP230 molecule [32]. By immunoblotting using these recombinant proteins, we found that the majority of the BP sera showed a strong reactivity with various domains of BP230, most frequently with the C-terminal domain. These results further confirmed that BP230 is the major autoantigen in BP [4,6]. The presence of multiple epitopes on BP230 for BP sera is consistent with the results in the previous studies [26,28,30]. In addition, there were two studies in which ELISA using recombinant proteins was developed [39,40].

In the present study, we developed more reliable ELISA using bacterial recombinant proteins of N-terminal (BP230-N) and C-terminal

Table 2 Specificity of BP230 ELISA and BP180 ELISA for control sera

	Pemphigus (n = 94)	Normal control	Specificity
BP230-ELISA	1.1% (1/94)	0% (0/109)	99.5% (202/203)
BP180 ELISA	0% (0/94)	1.5% (5/336)	98.8% (425/430)
BP230 + BP180	1.1% (1/94)		98.9% (93/94)

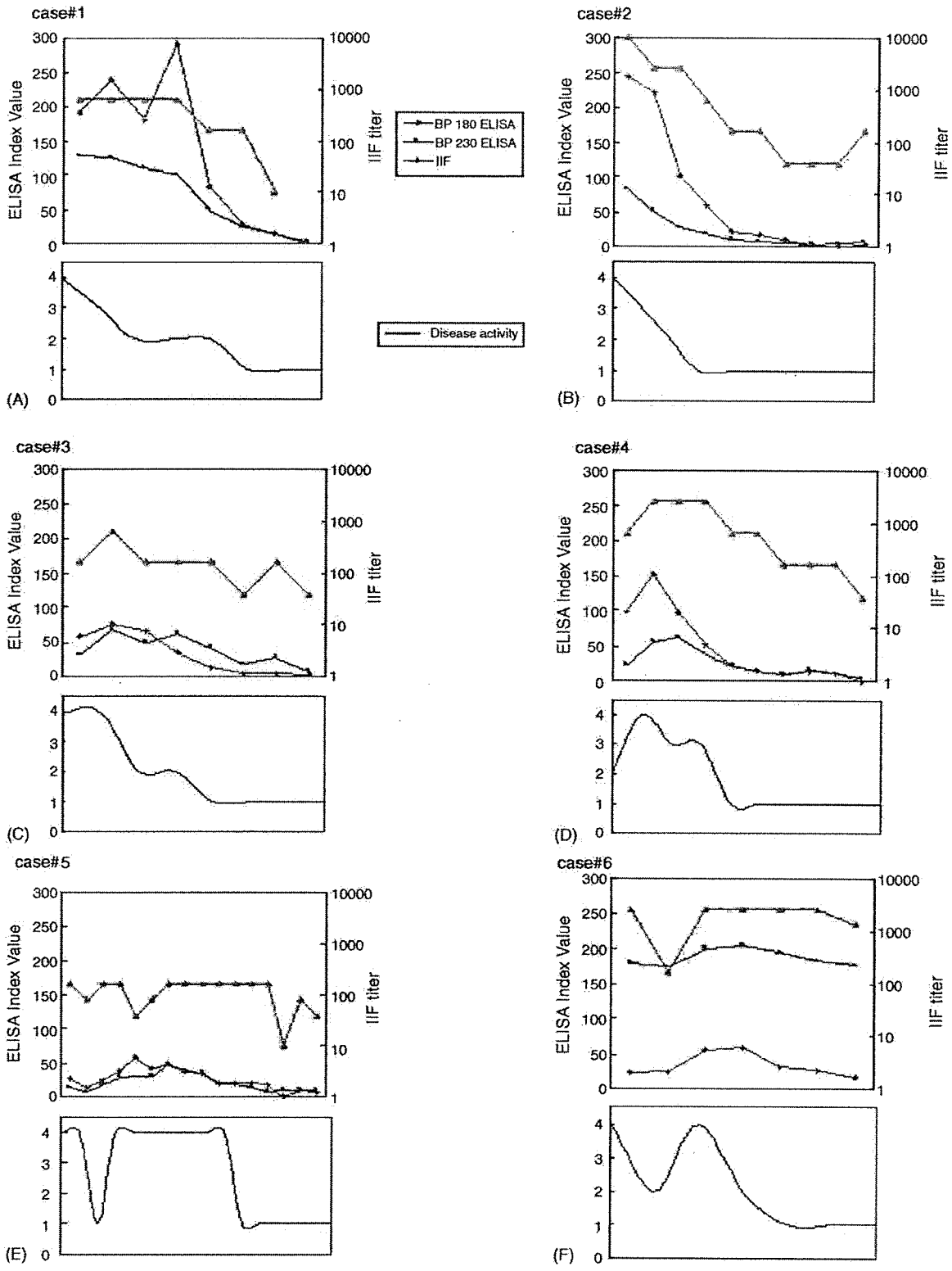

Fig. 4 (A–J) The index values of BP180 ELISA and BP230 ELISA, titers of indirect immunofluorescence (IIF) and disease activity in 10 BP patients. Note that the most active stages are shown as score 4 and remission stages are shown as score 1.

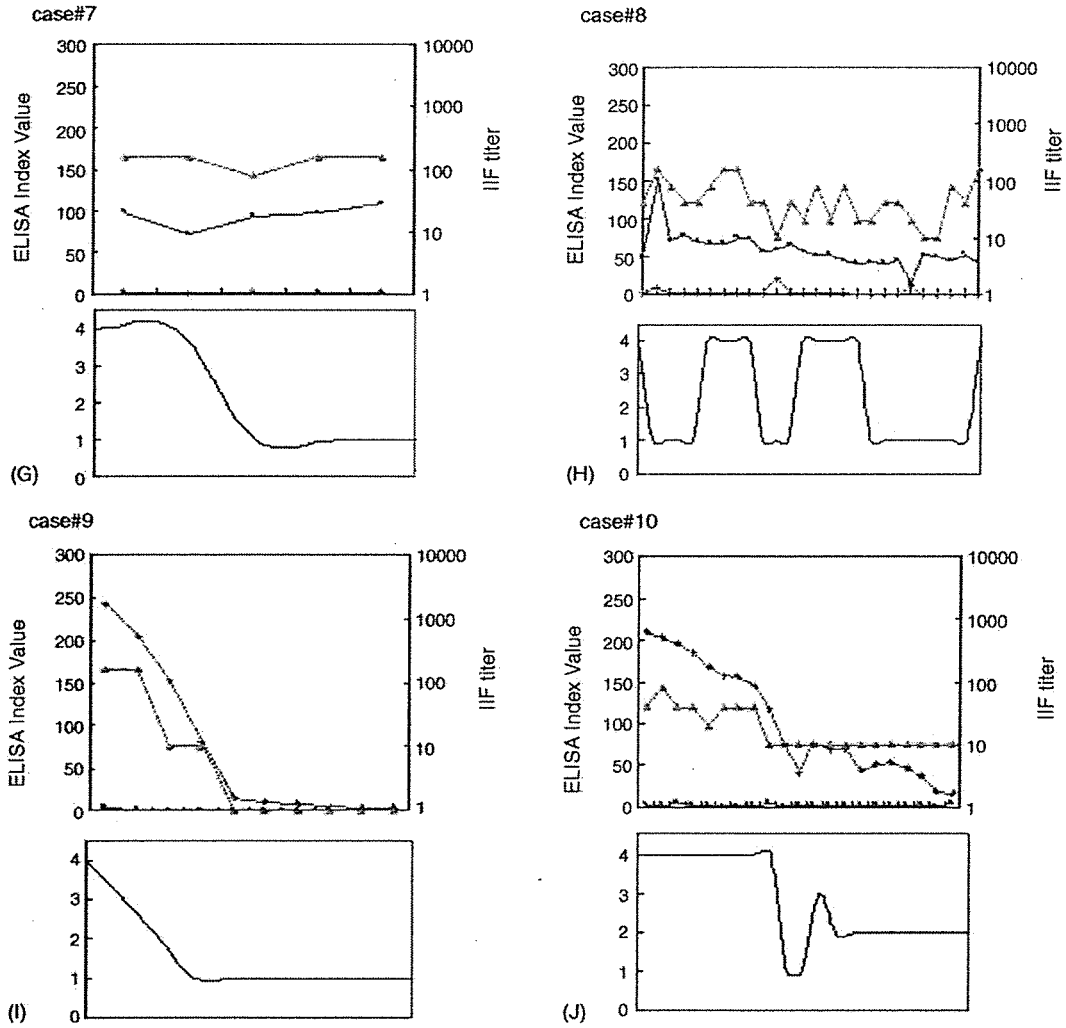

Figure 4. (*Continued*).

(BP230-C) domains of human BP230. Using the ELISA, we examined the sensitivity and specificity with a large number of BP sera as well as disease or normal control sera. The BP230 ELISA showed positive reactivity in 173 (72.4%) of the 239 BP sera. In contrast, only one serum of 94 pemphigus sera and 109 normal control sera was positive, indicating that the specificity of BP230 ELISA was 99.5%.

We also examined again the reactivity of BP180 ELISA for BP sera, pemphigus sera and normal control sera. The BP180 ELISA showed positive reactivity in 167 (69.9%) of the 239 BP sera. As reported before [24], the specificity of BP180 ELISA was 98.8%.

Most importantly, when the results of BP230 ELISA and BP180 ELISA were combined, the sensitivity raised as high as 97.1% (232/239). Therefore, using both the BP180 ELISA and BP230 ELISA, most of BP patient can be diagnosed.

Furthermore, by both the BP230 ELISA and BP180 ELISA, we examined multiple sera taken from 10 BP patients over the disease course and compared the results with those of indirect immunofluorescence. In five patients (cases 1–5), the scores of both BP230 and BP180 ELISAs fluctuated in parallel with disease activity. The indirect immunofluorescence titers also correlate roughly with the disease activity. In contrast, in three patients (cases 6–8) both the BP230 scores and the indirect immunofluorescence titers were high throughout the disease course, and did not correlate with disease activity. Interesting, in two of these three cases, the BP180 ELISA scores were negative in the whole disease course. In two BP patients (cases 9 and 10), the BP230 ELISA scores were totally negative, while the BP180 ELISA scores and the indirect immunofluorescence titers fluctuated in parallel with disease activity.

These results suggested that the index values of BP180 in general correlate to the disease severity and become negative in the patient in remission, although there are some cases showing negative results in the BP180 ELISA. In contrast, from the results of the BP230 ELISA, BP patients could be classified into three groups; i.e. (1) the cases showing a parallel fluctuation with disease activity, (2) the cases showing high scores constantly and (3) the cases showing negative results.

From these results, in the patients of group 1, because the scores of both BP230 and BP180 ELISAs correlate with disease activity, the indirect immunofluorescence titers, which are the sum of index values of both BP230 and BP180 ELISAs, also correlated with the disease activity. In patients of group 2, because the index values of BP230 ELISA are always high, the indirect immunofluorescence titers were also high throughout the disease course. In the patients of group 3, the BP230 ELISA scores are constantly negative, and both the BP180 ELISA scores and the indirect immunofluorescence titers were correlate with the disease activity. Therefore, in the cases of group 1, the BP230 ELISA index values can be used to determine the disease activity, while, in the cases of group 2, the BP230 index values do not reflect the disease course.

Kromminga et al. developed ELISA using five recombinant proteins of BP230 produced by baculovirus expression system. However, the sensitivity was 63% and specificity was 93% in their ELISA system [39]. Our ELISA is superior in both sensitivity and specificity. In addition, Thoma-Uszynski also developed ELISA for both BP180 and BP230 by baculovirus expression system [40]. However, the specificity of the BP230 ELISA was only 64.8%, although the sensitivity was 81.5%. In conclusion, our BP230 ELISA, when used in combination of BP180 ELISA, should be a useful tool for the diagnosis of BP in the future.

Acknowledgments

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EXPERIMENTAL HEMATOLOGY

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Human herpesvirus 6 impairs differentiation of monocytes to dendritic cells

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Objective. **Monocyte-derived dendritic cells (DCs) play important roles in the immune response against infections and malignancies. Human herpesvirus 6 (HHV-6) infects monocytes and is reactivated in immunodeficient patients. To clarify the mechanisms of HHV-6-induced immunodeficiency, we investigated the effect of HHV-6 infection on differentiation of monocytes to DCs.**

Methods. **Monocytes were inoculated with or without HHV-6 and then allowed to differentiate to myeloid DCs in culture medium containing granulocyte-macrophage colony-stimulating factor and interleukin (IL)-4. The expression of cell surface molecules on DCs and the capacity of the DCs for antigen capture were examined by flow cytometric analysis. Alteration of antigen-presenting capacity induced by HHV-6 infection was examined.**

Results. **The morphology of HHV-6-infected monocyte-derived DCs was distinctly different from that of the DCs derived from mock-infected monocytes. Although expression levels of DC-associated surface antigens, including CD80, CD83, and CD86, were significantly higher on HHV-6-infected monocyte-derived DCs than on DCs derived from mock-infected monocytes, antigen-presenting capacity was significantly lower in the former group. Addition of culture supernatant of HHV-6-infected monocytes resulted in suppression of the T-lymphocyte proliferative response, and anti-IL-10 neutralizing antibody partly inhibited this suppressive effect. The antigen-presenting capacity of DCs generated from a patient with severe HHV-6 reactivation was significantly lower than that of DCs generated from the same patient in the recovery phase.**

Conclusions. **HHV-6 infection induces immunodeficiency via impaired differentiation of DCs. These results present a new concept for the pathogenesis of HHV-6-induced immunodeficiency.** © **2006 International Society for Experimental Hematology. Published by Elsevier Inc.**

Reactivation of human herpesvirus 6 (HHV-6) occurs frequently in patients with immunodeficiency, such as hematopoietic stem cell and organ transplant recipients and those with acquired immune deficiency syndrome (AIDS) [1,2]. This reactivation causes various disorders, including lymphadenitis, pneumonitis, hepatitis, meningoencephalitis, retinitis, infectious mononucleosis-like disease, hemophagocytic syndrome, and drug-induced hypersensitivity syndrome (DIHS) [3–8]. HHV-6 was initially termed human B-lymphotropic virus because of its in vitro tropism for B lymphocytes [9]. However, it is now well known that HHV-6 exhibits tropism mainly for T lymphocytes and monocytes/macrophages and that various kinds of cell, including myeloid precursor cells, megakaryocytes, natural killer cells, fibroblasts, astrocytes, and hepatoma cells, are also susceptible to HHV-6 infection [10–15]. Various immunobiologic alterations of lymphocytes have been observed following infection. HHV-6A infection induces downregulation of CD3, resulting in impairment of T-lymphocyte activation via CD3/T-cell receptor complexes [16,17]. Upregulation of CD4, thus inducing susceptibility to human immunodeficiency virus-1 (HIV-1) infection, has been reported to occur in HHV-6A-infected $CD4^-$ T lymphocytes and natural killer cells [18–20]. HHV-6 infection of T lymphocytes reduces both interleukin (IL)-2 synthesis and the proliferative response to anti-CD3 monoclonal antibody (mAb) and phytohemagglutinin [21]. Transcriptional downregulation of CXCR4 (coreceptor for X4 HIV-1) is induced by HHV-6A and HHV-6B infections [22,23]. In addition to functional

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alteration of T lymphocytes, we have reported recently that HHV-6 infection results in impairment of antigen uptake and processing by dendritic cells (DCs) [24].

DCs are professional antigen-presenting cells (APCs) with a remarkable ability to stimulate naive T lymphocytes and generate memory T lymphocytes. Human DCs originating from hematopoietic stem cells can be divided into at least three subpopulations, including interstitial DCs residing in the skin and lymphoid organs and two subsets of blood DCs, the $CD11c^+$ myeloid and $CD11c^-$ plasmacytoid DCs [25]. Because circulating blood DCs are few and are difficult to maintain in culture, monocytes are commonly used as sources of myeloid DCs [26]. There is increasing evidence that maturation of DCs from monocytes may also be relevant in vivo [27,28].

HHV-6 latently infects monocytes [29] and is reactivated in immunodeficient patients. As mentioned above, HHV-6 alters the functions of various immunocompetent cells, and monocyte-derived DCs play important roles in the immune response against infections and malignancies. In light of these facts, clarification of the effects of HHV-6 infection on the differentiation of DCs from monocytes seems an important issue. We therefore investigated the morphologic and immunologic effects of HHV-6 infection on DC differentiation from peripheral blood monocytes.

Materials and methods

Isolation of monocytes and HHV-6 infection

The U1102 strain of HHV-6A and the Z29 strain of HHV-6B were grown in cord blood mononuclear cells that had been stimulated with phytohemagglutinin. Monocytes were isolated from peripheral blood mononuclear cells (PBMCs) of healthy individuals using anti-CD14 mAb-coated magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). In some experiments, monocytes were isolated from PBMCs of a patient with DIHS, in whom HHV-6 had been severely reactivated, in the acute and recovery phases. The patient's PBMCs were frozen in a liquid nitrogen tank until use. The purity of the monocytes, as determined by their morphology and by flow cytometric analysis using anti-CD14 mAb, was more than 95%. Monocytes were inoculated with HHV-6 at an approximate multiplicity of infection of one 50% tissue culture infective doses.

Differentiation of mock-infected and HHV-6-infected monocytes to DCs

Differentiation of DCs from monocytes was induced, as described previously [30]. Briefly, monocytes were inoculated by incubation with mock stock or HHV-6 stock at 37°C for 2 hours and then washed once. Mock-infected and HHV-6-infected monocytes were then suspended in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), 500 U/mL recombinant human IL-4 (Genzyme, Boston, MA, USA), and 800 U/mL recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; Kirin Brewery, Tokyo, Japan) and cultured in a 5% CO_2 incubator at 37°C. On day 2 or 3 of incubation, half of the medium was exchanged for fresh culture medium supplemented with IL-4 and GM-CSF, and culture was continued. On day 5, the cells were harvested and used as monocyte-derived immature myeloid DCs. Mature myeloid DCs used as the control cells for phenotypic analysis were generated from immature myeloid DCs by adding tumor necrosis factor-α, as described previously [24].

HHV-6 replication in monocyte-derived DCs

Replication of HHV-6 in monocyte-derived DCs was examined by detecting mRNA for the HHV-6 immediate-early and late (*U83*) genes by reverse transcription-polymerase chain reaction (RT-PCR), as described previously [15,31]. Briefly, total RNAs were extracted from HHV-6-inoculated or mock-infected cells, and cDNA was synthesized by reverse transcription with Moloney murine leukemia virus reverse transcriptase. Amplification of the cDNAs by PCR was performed using the following primers: immediate early gene, 5′-TTCTCCAGATGTGCCAGGGAAATCC-3′ and 5′-CATCATTGTTATCGCTTTCACTCTC-3′; late gene, 5′-GTCGACCATGTTCATTTGGCTTTTTATTGTT-3′ and 5′-ATGAATTCTCATGATTCTTTGTCTAATTTC-3′. The expected lengths of the amplified cDNA sequences for the HHV-6 immediate early gene and late gene were 553 bp and 345 bp, respectively. Amplification of cDNA for the β-actin gene was also performed as the control for RT-PCR, using the following primers: 5′-TCCTGTGGCATCCACGAAACT-3′ and 5′-GAAGCATTTGCGGTGGACGAT-3′.

Quantitative real-time PCR for the HHV-6 genome was performed as reported previously [32]. The PCR primers and probe for HHV-6 DNA were selected from the *U31* gene coding a large tegument protein. The upstream primer was 5′-TTTGCAG TCATCACGAT-CGG-3′ and the downstream primer was 5′-AGAGCGACAAATTGGAGGTTTC-3′. A fluorogenic probe, 5′-AGCCACAGCAGCCATCTACATCTGTCAA-3′, was located between the primers. The PCR reaction was performed using a TaqMan PCR kit (PE Applied Biosystems, Foster City, CA, USA), as described previously [32].

Flow cytometric analysis of HHV-6 antigen expression in DCs

Simultaneous detection of surface CD80 and intracellular HHV-6 antigen expression was performed as follows. Mock-infected and HHV-6-infected monocyte-derived DCs were incubated with a fluorescein isothiocyanate (FITC)-conjugated anti-CD80 mAb (Immunotech, Marseilles, France). To further detect intracellular HHV-6 antigen expression, cells were then fixed with 3.0% formaldehyde, permeabilized with 0.05% saponin, and incubated with mAb against the HHV-6 101-kDa virion protein (Chemicon International, Temecula, CA, USA), followed by staining with phycoerythrin-conjugated goat anti-mouse immunoglobulin (Ig) G (Organon Teknika, West Chester, PA, USA). After being washed, the cells were analyzed with a FACSCalibur system (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) equipped with CellQuest software (Becton Dickinson Immunocytometry Systems).

Morphology of mock-infected and HHV-6-infected monocyte-derived DCs

The morphologic differences between mock-infected and HHV-6-infected monocyte-derived DCs were examined under an inverted microscope. Cells fixed on the glass slides by cytospin were stained with May-Giemsa solution. Transmission electron microscopy was performed as described previously [15]. Briefly, the cells were fixed with 2.0% glutaraldehyde in 0.1 M phosphate buffer

(pH 7.4), postfixed with 1% osmium tetroxide, and gradually dehydrated. Samples were embedded in Epon 812, sectioned, stained with uranyl acetate and lead citrate, and examined with a JEM-1230 electron microscope (JEOL, Peabody, MA, USA).

Flow cytometric analysis of cell surface molecule expression

The expression of cell surface molecules on immature and mature DCs differentiated from mock-infected and HHV-6-infected monocytes was examined by flow cytometric analysis using the following mAbs: anti-CD1a (Immunotech), anti-CD40 (PharMingen, San Diego, CA, USA), anti-CD44 (PharMingen), anti-CD80 (Immunotech), anti-CD83 (Immunotech), anti-CD86 (Immunotech), anti-CD209 (DC-SIGN) (Immunotech), anti-HLA class I (PharMingen), and anti-HLA-DR (PharMingen). To block nonspecific binding of mAbs, cells were preincubated with human Ig (Bayer AG, Leverkusen, Germany) at a concentration of 1 mg/mL for 30 minutes before the mAbs were added. After washing, the cells were analyzed as described above.

Flow cytometric analysis of endocytosis

The capacity of the monocyte-derived DCs for antigen capture was examined quantitatively by flow cytometric analysis of endocytosis, as described previously [33]. Briefly, mock-infected and HHV-6-infected monocyte-derived immature and mature DCs were suspended in RPMI 1640 medium supplemented with 10% FCS at 37°C or 4°C. Lucifer yellow CH (Molecular Probes, Eugene, OR, USA) or lysine-fixable FITC-dextran (molecular weight = 40,000; Sigma, St. Louis, MO, USA) was added at a final concentration of 1 mg/mL. The cells were incubated for 30 minutes and washed four times with cold phosphate-buffered saline containing 1% FCS and 0.01% NaN_3 and were analyzed using a FACSCalibur. The staining of cells that had been incubated at 4°C was analyzed as a control.

Presentation of alloantigen to T lymphocytes by monocyte-derived DCs

The allostimulatory capacity of mock-infected and HHV-6-infected monocyte-derived DCs was examined as follows. PBMCs were isolated from donors whose HLA types were nonidentical to those of the monocyte donors. PBMCs (1×10^5) and various numbers of mock-infected and HHV-6-infected monocyte-derived DCs that had been treated with mitomycin C (MMC; Kyowa Hakko, Tokyo, Japan) were cocultured in flat-bottomed microtiter wells each containing 0.2 mL RPMI 1640 medium supplemented with 10% human AB-type serum. The cells were then incubated for 6 days. For the final 16 hours of incubation, 1 μCi [^{3}H]TdR (New England Nuclear, Boston, MA, USA) was added to each well, and the incorporation of [^{3}H]TdR was determined by liquid scintillation counting.

Presentation of exogenous virus antigen to T lymphocytes by monocyte-derived DCs

The exogenous antigen-presentation capacity of mock-infected and HHV-6-infected monocyte-derived DCs was examined using a modification of a previously reported method [34]. Briefly, herpes simplex virus type 1 (HSV-1) antigen was prepared by ultraviolet light irradiation of the virus. PBMCs were isolated from donors who were seropositive for HSV. T lymphocytes were purified from PBMCs by passage through nylon-wool columns and DCs were generated from monocytes from the same donors of T lymphocytes, as described above. T lymphocytes (1×10^5) and various numbers of MMC-treated mock-infected or HHV-6-infected monocyte-derived DCs in 0.2 mL RPMI 1640 medium supplemented with 10% human AB-type serum were seeded in flat-bottomed microtiter wells, to which 0.02 mL virus antigen or control antigen prepared from mock-infected cells was added at the optimal dilution. The cells were cultured for 6 days and the incorporation of [^{3}H]TdR was determined, as described above.

Presentation of peptide antigen to peptide-specific T lymphocytes by monocyte-derived DCs

A chronic myelogenous leukemia-associated fusion protein, the BCR-ABL-specific $CD4^+$ T-lymphocyte clone MY-1, was generated as described previously [35]. MY-1 induces a BCR-ABL fusion peptide (ATGFKQSSKALQRPVAS)-specific and HLA-DRB1*0901-restricted proliferative response. Proliferative response assay of MY-1 was performed using a modification of a previously described method [35]. Briefly, monocytes were isolated from HLA-DRB1*0901-positive donors. MY-1 cells (5×10^4) and various numbers of MMC-treated mock-infected or HHV-6-infected monocyte-derived DCs in 0.2 mL RPMI 1640 medium supplemented with 10% human AB-type serum were seeded into flat-bottomed microtiter wells, to which BCR-ABL peptide was added at 10 μM. The cells were cultured for 3 days and the incorporation of [^{3}H]TdR was determined as described above.

Effect of culture supernatant of mock-infected and HHV-6-infected monocytes on T-cell proliferation

To determine whether soluble suppressor factors of T-lymphocyte activation were produced by HHV-6-infected monocytes, the culture supernatants of mock-infected and HHV-6-infected monocytes were added to MY-1 cells and their proliferation in response to stimulation with BCR-ABL peptide was examined as follows. The culture supernatants of mock-infected and HHV-6-infected monocytes were collected after 3 days of infection and irradiated with ultraviolet to inactivate any HHV-6 produced. The proliferative response of MY-1 to stimulation with BCR-ABL peptide was examined as described above, with a slight modification. Briefly, MY-1 cells (5×10^4) and MMC-treated HLA-DRB1*0901-positive monocyte-derived DCs (2×10^4) in 0.15 mL RPMI 1640 medium supplemented with 10% human AB-type serum and 0.05 mL of culture supernatant of the mock-infected or HHV-6-infected monocytes were seeded into flat-bottomed microtiter wells, to which BCR-ABL peptide was added at 10 μM. The cells were cultured for 3 days and the incorporation of [^{3}H]TdR was determined as described above. In some experiments, the culture supernatants were incubated with anti-IL-10 neutralizing antibody (R&D Systems, Minneapolis, MN, USA) or control antibody at the optimal concentration for 1 hour before being added to the culture wells. In preliminary experiments, more than 90% of the biologic activity of IL-10 was inactivated after incubation with the neutralizing antibody (data not shown).

Results

Replication of HHV-6 in monocyte-derived DCs

Replication of HHV-6 in monocyte-derived DCs was examined by RT-PCR for HHV-6 immediate-early and late genes. RT-PCR analysis clearly showed the presence of

mRNAs for the HHV-6 immediate-early and late genes in monocyte-derived DCs that had been inoculated with HHV-6B and cultured for 5 days (Fig. 1A). Quantitative real-time PCR analysis showed a large amount of HHV-6 copy in HHV-6B-inoculated monocyte-derived DCs 3 and 5 days after inoculation (Fig. 1B). In addition, flow cytometric analysis revealed the presence of HHV-6 antigen in these DCs (Fig. 1C). Moreover, infectious HHV-6 was recovered from them (data not shown). Similarly, replication of HHV-6A in monocyte-derived DCs was also detected (data not shown). These data demonstrate that both HHV-6A and HHV-6B can infect and replicate in monocyte-derived DCs. Because most of the HHV-6-infected monocyte-derived DCs were alive 5 days after inoculation, DCs harvested 5 days after HHV-6 inoculation were used in the following experiments.

Morphology of mock-infected and HHV-6-infected DCs

The morphologic differences between mock-infected and HHV-6-infected monocyte-derived DCs are shown in Figure 2. Inverted microscopy of mock-infected monocyte-derived DCs revealed the typical DC appearance with many dendritic processes; however, HHV-6-infected monocyte-derived DCs appeared to lack these processes and were instead round (Fig. 2A). These distinct morphologic differences were also detected by cytospin preparation with May-Giemsa staining (Fig. 2B) and transmission electron microscopy (Fig. 2C). Both DCs derived from mock-infected and HHV-6-infected monocytes were not adherent to plastic (data not shown).

Flow cytometric analysis of surface molecule expression on mock-infected and HHV-6-infected monocyte-derived DCs

We next examined alterations of surface molecule expression on HHV-6-infected monocyte-derived DCs. Upregulation of CD40, CD80, CD83, CD86, HLA class I, and HLA class II molecules was detected in HHV-6B-infected monocyte-derived immature DCs (Fig. 3). In contrast, expression level of CD209 (DC-SIGN) was decreased. On the other hand, surface molecule expression was not significantly different between mock-infected and HHV-6B-infected monocyte-derived mature DCs, except for CD209. Similar alterations of surface molecule expression were detected in HHV-6A-infected monocyte-derived DCs (data not shown). Inoculation of monocytes with ultraviolet-inactivated HHV-6 did not affect surface molecule expression on differentiated DCs (data not shown), suggesting that replication of HHV-6 is necessary to induce alterations in the expression of these molecules.

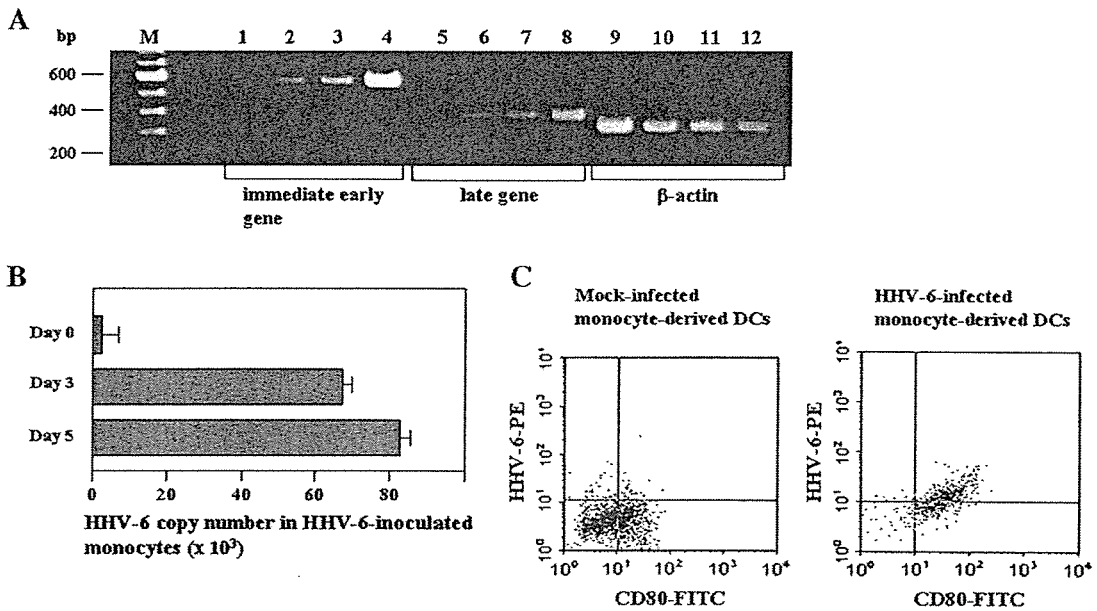

Figure 1. Replication of HHV-6 in monocyte-derived DCs. **(A):** Expression of HHV-6 mRNA in DCs. cDNAs synthesized from mock-infected monocyte-derived DCs (lanes 1, 5, and 9), HHV-6B-infected monocyte-derived DCs on day 3 after inoculation (lanes 2, 6, and 10), HHV-6B-infected monocyte-derived DCs on day 5 after inoculation (lanes 3, 7, and 11), and HHV-6B-infected cord blood mononuclear cells (lanes 4, 8, 12) were amplified using primers corresponding to the HHV-6 immediate-early and late genes and primers corresponding to the β-actin gene. Lane M shows marker DNAs. **(B):** Quantitative real-time PCR for the HHV-6 genome in monocyte-derived DCs. Mock-infected and HHV-6B-inoculated monocytes were cultured in the presence of GM-CSF and IL-4 for 3 or 5 days. DNAs were extracted from samples, and copy numbers of HHV-6 were estimated as described in Materials and Methods. **(C):** Flow cytometric analysis of HHV-6 antigen expression in monocyte-derived DCs. Mock-infected and HHV-6B-inoculated monocytes were cultured in the presence of GM-CSF and IL-4 for 5 days. Expression of cell surface CD80 and intracellular HHV-6 antigen was then examined by two-color flow cytometry.

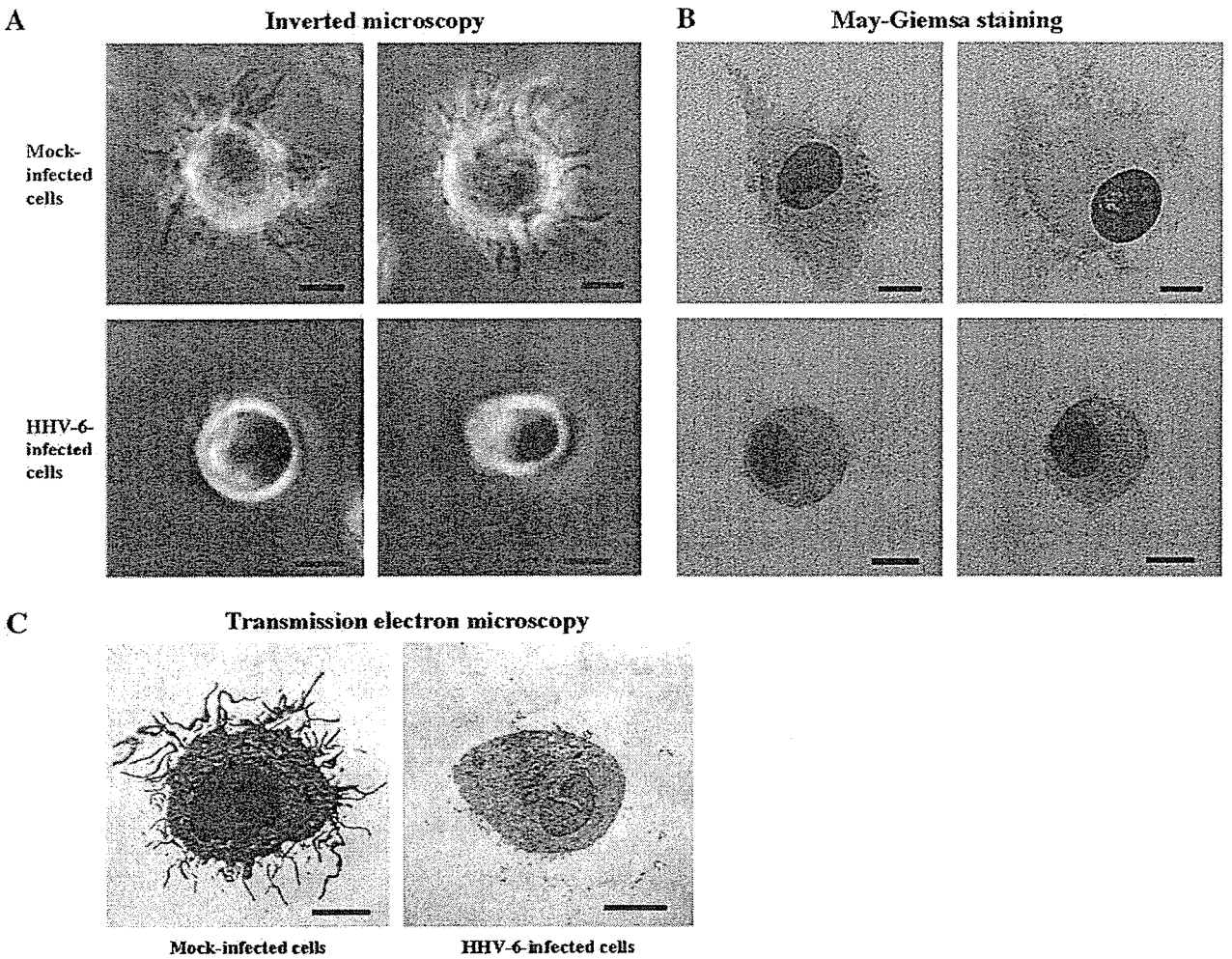

Figure 2. Morphologies of mock-infected and HHV-6-infected monocyte-derived DCs. Mock-infected and HHV-6B-inoculated monocytes were cultured in the presence of GM-CSF and IL-4 for 5 days. Morphologies of monocyte-derived DCs were examined by inverted microscopy **(A)**, after May-Giemsa staining of cytospin preparations **(B)**, and transmission electron microscopy (×1500) **(C)**. The bars represent 5 μm.

Reduced endocytosis by HHV-6-infected monocyte-derived DCs

Because uptake of antigens is an important function of DCs, we investigated whether HHV-6 infection affects the antigen-capture capacity of monocyte-derived DCs. Figure 4 shows the endocytosis profiles of mock-infected, HHV-6A-infected, and HHV-6B-infected monocyte-derived immature and mature DCs. The level of endocytosis mediated by HHV-6-infected monocyte-derived immature DCs was significantly lower than that mediated by mock-infected monocyte-derived immature DCs.

Reduced allostimulatory capacity of HHV-6-infected monocyte-derived DCs

The antigen-presentation capacity of mock-infected and HHV-6-infected monocyte-derived DCs was first examined by determining the stimulatory capacity of alloantigens. Allogeneic T lymphocytes isolated from donors whose HLA class I and class II types were nonidentical with those of the monocyte donors were cocultured with various numbers of monocyte-derived DCs. The allostimulatory capacity of HHV-6-infected monocyte-derived DCs was significantly lower than that of DCs derived from mock-infected monocytes (Fig. 5). The same experiments were performed three times and similar data were obtained (data not shown). These data indicate that HHV-6 infection alters the antigen-presentation capacity of monocytes.

Reduced viral antigen-stimulatory capacity of HHV-6-infected monocyte-derived DCs

We next examined the capacity of mock-infected and HHV-6-infected monocyte-derived DCs to present exogenous viral antigen. Exogenous antigens were at first captured, processed, and then presented to mainly antigen-specific $CD4^+$ T lymphocytes by the APCs, in the context of major histocompatibility complex class II molecules. Figure 6

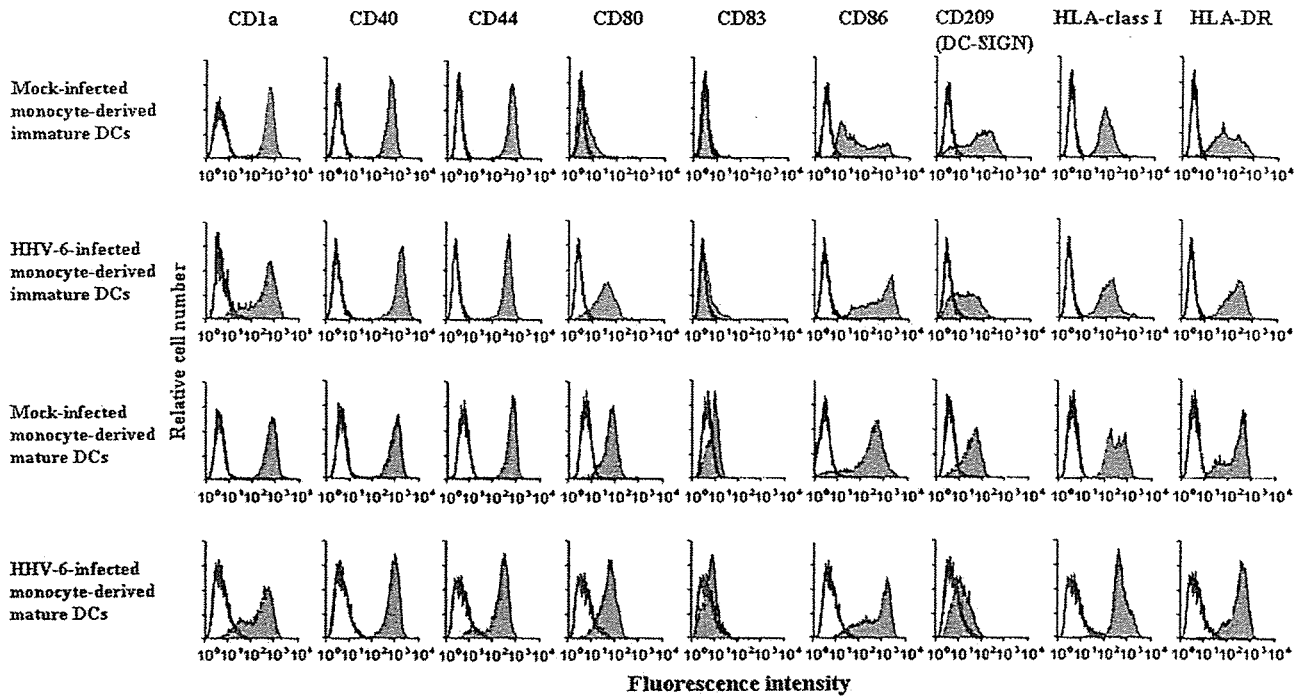

Figure 3. Flow cytometric analysis of mock-infected and HHV-6-infected monocyte-derived immature and mature DCs. Cell surface expression levels of CD1a, CD40, CD44, CD80, CD83, CD86, CD209 (DC-SIGN), HLA class I, and HLA-DR on the four types of DCs were measured by flow cytometry.

shows the proliferative responses of autologous T lymphocytes to HSV-1 antigen in the presence of mock-infected and HHV-6-infected monocyte-derived DCs. In the same manner as the response to alloantigens, the proliferative response of T lymphocytes to exogenous HSV-1 antigen was significantly lower when HHV-6A-infected or HHV-6B-infected monocyte-derived DCs were used as APCs than when DCs derived from mock-infected monocytes were used. The same experiments were performed three times, and similar data were obtained (data not shown). These data indicate that the pathway of presentation of exogenous antigens in monocyte-derived DCs is also impaired by HHV-6 infection.

Reduced peptide antigen-stimulatory capacity of HHV-6-infected monocyte-derived DCs

We examined further the presentation capacity of a 17-mer peptide that could be presented to peptide-specific $CD4^+$ T lymphocytes unnecessarily by being captured and processed by APCs. As we reported previously, the BCR-ABL fusion peptide-specific $CD4^+$ T-lymphocyte clone, MY-1, proliferates in response to stimulation with a BCR-ABL peptide in the presence of HLA-DRB1*0901-positive APCs. The proliferative response of MY-1 to the synthetic peptide in the presence of mock-infected or HHV-6-infected HLA class II–matched monocyte-derived DCs is shown in Figure 7. The degree of proliferative response mediated by MY-1 to the synthetic peptide appeared to be slightly lower when HHV-6-infected monocyte-derived DCs were used as APCs than when mock-infected monocyte-derived DCs were used. Taken together, the data presented in Figures 3, 4, 5, and 6 suggest strongly that in addition to alterations of antigen capture and antigen processing capacities, suppressor factor(s) may be produced by HHV-6-infected monocytes.

Production of suppressor factor(s) by HHV-6-infected monocytes

The data shown above suggest that the dysfunction of antigen presentation by HHV-6-infected monocyte-derived DCs is due to the production of immunosuppressive factor(s) in addition to the impairment of uptake and processing of antigens. We therefore verified that T-lymphocyte activation is suppressed by factor(s) produced from HHV-6-infected monocytes. Because IL-10 is a strongly immunosuppressive cytokine and is produced by monocytes, our investigation was focused on IL-10. The concentrations of IL-10 in the culture supernatants of mock-infected and HHV-6-infected monocytes were less than 20 pg/mL ($n = 4$) and 102 ± 10 pg/mL ($n = 4$), respectively. Adding culture supernatant of HHV-6-infected monocytes resulted in a reduced proliferative response of the BCR-ABL peptide-specific $CD4^+$ T-cell clone, MY-1, to stimulation with the peptide (Fig. 8). This inhibitory effect of the culture supernatant appeared to be partly inhibited by anti-IL-10 neutralizing antibody. These data indicate that

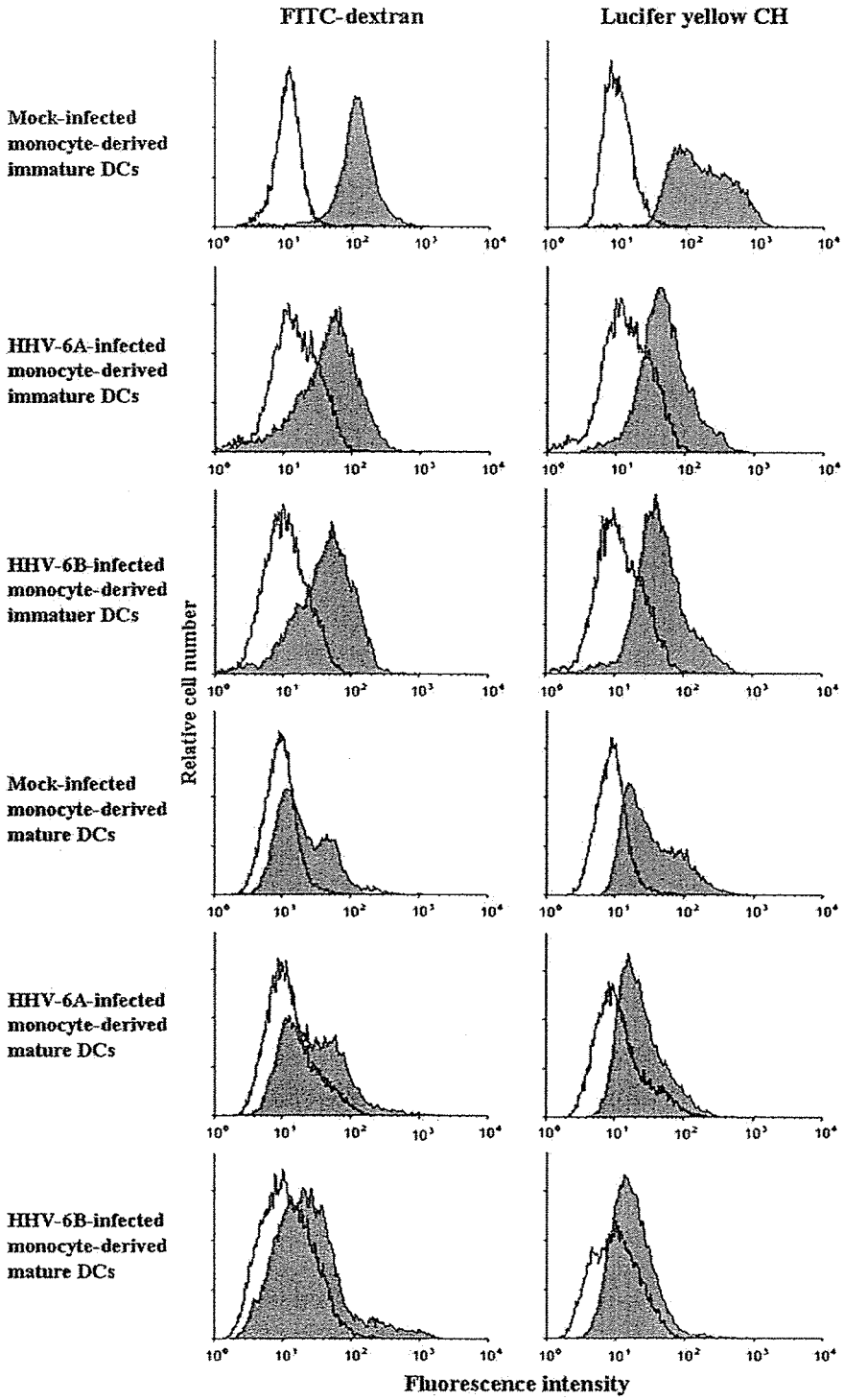

Figure 4. Flow cytometric analysis of endocytosis mediated by mock-infected, HHV-6A-infected, and HHV-6B-infected monocyte-derived immature and mature DCs. The six types of DCs were incubated in a medium containing FITC-dextran or Lucifer yellow CH at 37°C for 30 minutes (shaded histograms). The controls were evaluated by incubating cells in a medium containing FITC-dextran or Lucifer yellow CH at 4°C (clear histograms).

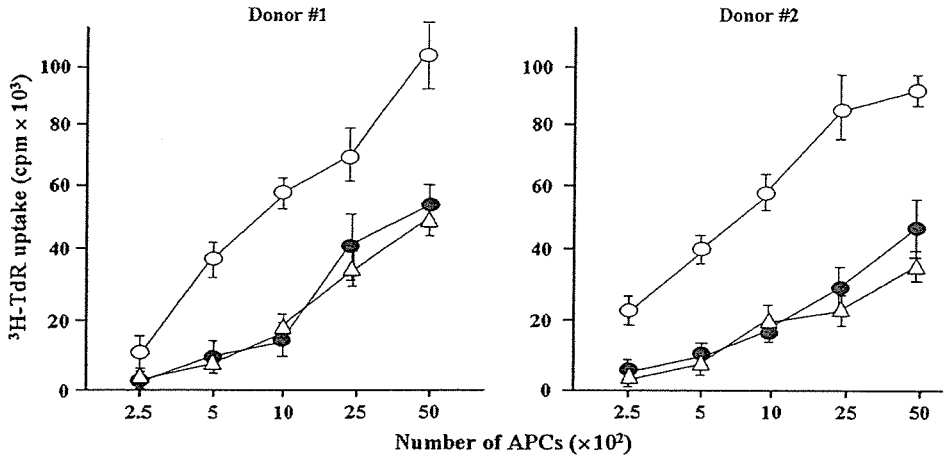

Figure 5. Presentation of alloantigens to allogeneic T lymphocytes by mock-infected and HHV-6-infected monocyte-derived DCs. Incorporation of [^{3}H]TdR into allogeneic T lymphocytes was determined in the presence of mock-infected monocyte-derived DCs (open circles), HHV-6A-infected monocyte-derived DCs (open triangles), or HHV-6B-infected monocyte-derived DCs (closed circles). Data represent means ± SDs of quadruplicate wells.

immunosuppressive factors, including IL-10, are produced by HHV-6-infected monocytes.

Impairment of antigen presentation by monocyte-derived DCs in a patient with HHV-6 reactivation

As mentioned above, HHV-6 infection of monocytes resulted in severe alteration of their antigen-presenting capacity in vitro. To confirm that this phenomenon also occurs in vivo, we examined the antigen-presenting capacity of monocyte-derived DCs isolated from a patient with DIHS who had severe HHV-6 viremia. Because the number of monocytes obtained from the patient was limited and the patient was negative for HLA-DR9, which is the restriction element of the peptide-specific T-cell clone MY-1, only the capacity to present alloantigen could be examined. Monocyte-derived DCs were generated from this patient during both HHV-6 viremia, when the number of copies of the HHV-6 genome in the PBMCs amounted to 11,000/μg DNA, and recovery, when the number of copies was 0/μg DNA. The allostimulatory capacity of monocyte-derived DCs isolated from the patient during HHV-6 viremia was significantly lower than that of mock-infected monocyte-derived DCs isolated from the patient in the recovery phase

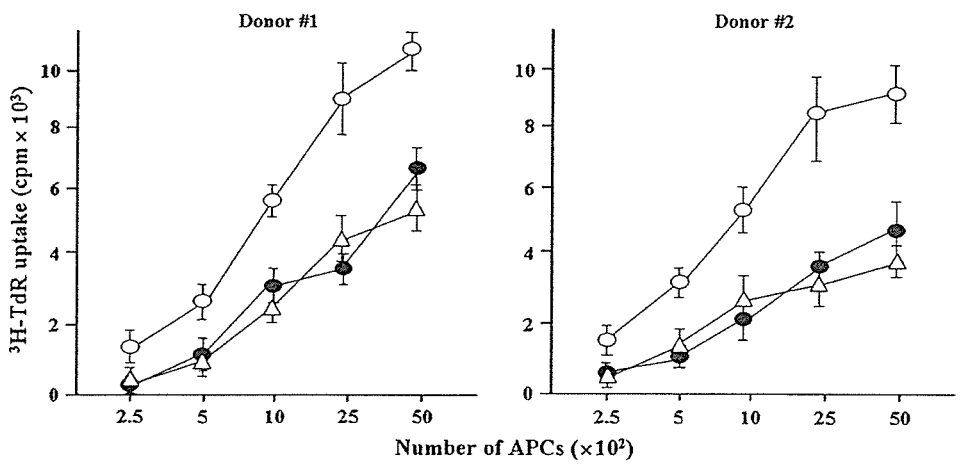

Figure 6. Presentation of exogenous HSV antigen to autologous T lymphocytes by mock-infected and HHV-6-infected monocyte-derived DCs. Incorporation of [^{3}H]TdR into autologous T lymphocytes was determined in the presence of mock-infected monocyte-derived DCs (open circles), HHV-6A-infected monocyte-derived DCs (open triangles), or HHV-6B-infected monocyte-derived DCs (closed circles) with or without viral antigen. The antigen-specific proliferative response of T lymphocytes was measured by subtracting the count obtained from preparations with no antigen stimulation from that obtained with HSV antigen stimulation. Data represent means ± SDs of quadruplicate wells.

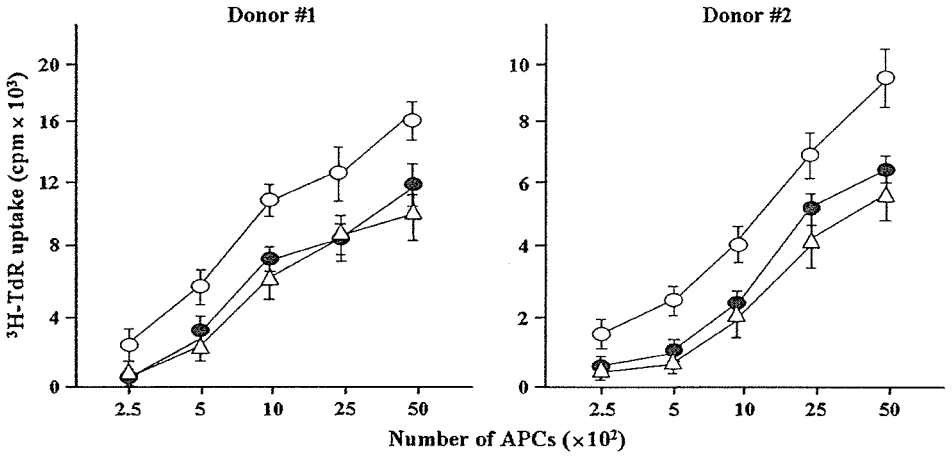

Figure 7. Presentation of peptide to a peptide-specific T-lymphocyte clone by mock-infected and HHV-6-infected monocyte-derived DCs. Incorporation of [^{3}H]TdR into BCR-ABL peptide-specific T-lymphocyte clones was determined in the presence of mock-infected monocyte-derived DCs (open circles), HHV-6A-infected monocyte-derived DCs (open triangles), or HHV-6B-infected monocyte-derived DCs (closed circles), with or without the BCR-ABL peptide at a concentration of 10 μM. Data represent means ± SDs of quadruplicate wells.

(Fig. 9). These data suggest strongly that impairment of antigen presentation by monocyte-derived DCs certainly occurs in vivo as well as in vitro.

Discussion

Because the induction of altered DC function by viral infection is an important issue in the pathogenesis of virus-induced immunodeficiency, studies focusing on the effects of viral infection on the phenotype and function of DCs have been reported. Various viruses affect the maturation of DCs. For example, vaccinia virus inhibits DC maturation, thus reducing the capacity of DCs to stimulate T lymphocytes [36]. A similar phenomenon has also been demonstrated in HSV-infected DCs [37]. Although some

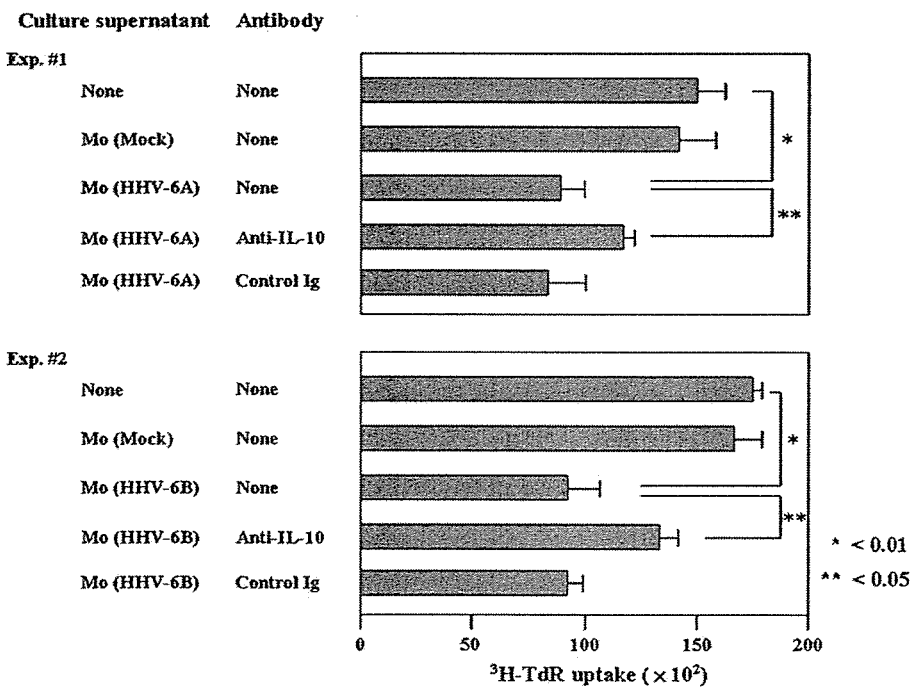

Figure 8. Effects of culture supernatants of mock-infected and HHV-6-infected monocytes on proliferative response of T-lymphocyte clone. Culture supernatants of mock-infected and HHV-6-infected monocytes were collected 3 days after infection. The proliferative response of BCR-ABL-specific T-lymphocyte clone to stimulation with BCR-ABL peptide in the presence or absence of culture supernatant was examined as described above. In some experiments, the culture supernatants were incubated with anti-IL-10 neutralizing antibody before being added to the culture wells. Data represent means ± SDs of quadruplicate wells.

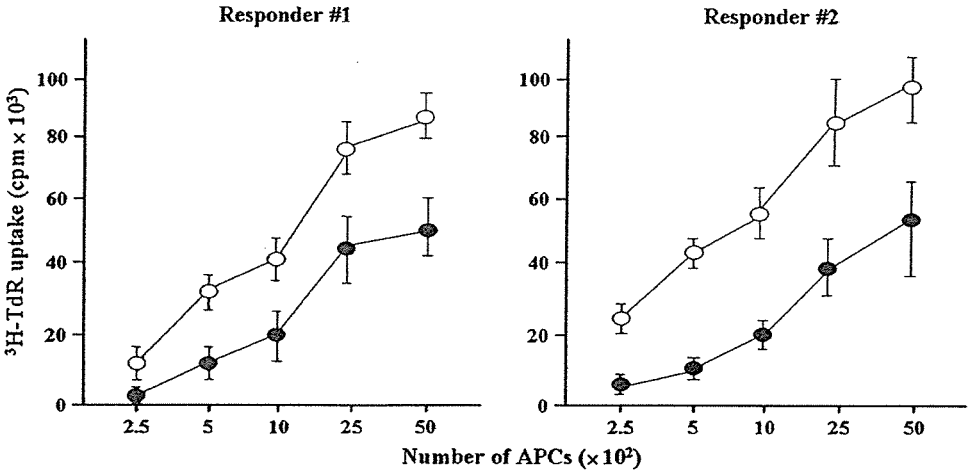

Figure 9. Presentation of alloantigens to allogeneic T lymphocytes by monocyte-derived DCs from a patient with DIHS. Monocyte-derived DCs were generated from PBMCs of the DIHS patient during the HHV-6 viremia and recovery phases. Incorporation of [^{3}H]TdR into allogeneic T lymphocytes was determined in the presence of monocyte-derived DCs generated during HHV-6 viremia (closed circles) or the recovery phase (open circles). Data represent means ± SDs of quadruplicate wells.

viruses impair the process of maturation of DCs, other viruses have been shown to drive DC maturation. Measles virus infection of immature DCs induces DC maturation and dysfunction of their capacity to stimulate T lymphocytes [38–40]. It has also been reported that dengue virus infection of immature DCs leads to their maturation [41]. We reported recently that HHV-6 infection of immature DCs results in impairment of antigen-presentation capacity, despite phenotypic maturation [24].

Because monocytes are the major source of myeloid DCs and HHV-6 latently infects monocytes, we investigated the morphologic and functional effects of HHV-6 infection on the differentiation of monocytes to DCs. Consequently, we found the following evidence. First, the morphology of DCs differentiated from HHV-6-infected monocytes is distinctly different from that of DCs differentiated from mock-infected monocytes. Second, the levels of expression of DC-associated molecules, such as CD80, CD83, and CD86, are higher on HHV-6-infected monocyte-derived DCs than on mock-infected monocyte-derived DCs. Third, the antigen-uptake capacity of HHV-6-infected monocyte-derived DCs is lower than that of DCs derived from mock-infected monocytes. Fourth, the capacity to present antigens such as alloantigen, exogenous viral antigen, and epitope peptide is lower in HHV-6-infected monocyte-derived DCs than in mock-infected monocyte-derived DCs, and DCs derived from HHV-6-infected monocytes produce immunosuppressive factors, including IL-10. Finally, DCs generated from monocytes isolated from a patient with severe HHV-6 reactivation showed impairment of antigen-presentation capacity, suggesting strongly that HHV-6-induced dysfunction of monocyte-derived DCs occurs in patients with HHV-6 infection.

An interesting finding of our study is the morphology of HHV-6-infected monocyte-derived DCs. Although mock-infected monocytes had the typical DC appearance after incubation with GM-CSF and IL-4, as described previously [29], DCs derived from HHV-6-infected monocytes were round and lacked dendritic processes. It has been reported that syncytium formation of DCs is induced by measles virus infection [38–40]; however, the morphologic changes induced in DCs by infection with other viruses have not been precisely reported. Although the mechanism of alteration of the morphology of HHV-6-infected monocyte-derived DCs is unknown, active replication of HHV-6 may be required to induce such changes, because DCs derived from monocytes inoculated with inactivated HHV-6 showed the typical DC appearance (data not shown).

Our most important finding is that antigen-presenting capacity, which is the professional function of DCs, is severely impaired in HHV-6-infected monocyte-derived DCs. Impairment of the antigen-presenting capacity of DCs has been reported in various viral infections. In most studies reported previously, only the alloantigen-stimulatory capacity of DCs to allogeneic T lymphocytes has been used to evaluate antigen-presenting capacity. We performed a precise examination of the antigen-presenting capacity of DCs using exogenous viral antigen and a peptide antigen unnecessarily captured and processed by DCs. Moreover, we also examined the capacity of DCs for antigen capture by flow cytometric analysis of endocytosis. Consequently, we found that the antigen-presenting capacity of HHV-6-infected monocyte-derived DCs is impaired in several ways. First, antigen-capture capacity is decreased. Second, processing of endogenous and exogenous antigens may also be impaired. Third, the capacity to present peptide that can

bind to HLA molecules unnecessarily by being captured and processed by DCs is also decreased, despite normal expression of HLA and costimulatory molecules, suggesting strongly that immunosuppressive factor(s) may be produced by HHV-6-infected monocyte-derived DCs.

Because IL-10 is one of the major immunosuppressive cytokines and is known to be produced by virus-infected DCs, we further studied the mechanisms of DC dysfunction induced by HHV-6 infection, focusing on IL-10. Production of IL-10 was detected in the culture supernatant of HHV-6-infected monocytes as described previously [42], and adding anti-IL-10 antibody resulted in partial reduction of the inhibitory effect of culture supernatant of HHV-6-infected monocytes on the proliferative response of T lymphocytes. These data indicate that production of IL-10 is one of the causes of the monocyte-derived DC dysfunction induced by HHV-6 infection. Li and colleagues reported that HHV-6 infection induced the production of IL-10 and IL-12 by monocytes. Expression of IL-12 mRNA decreased with accumulation of IL-10 mRNA, and production of IL-12 was increased when anti-IL-10 mAb was added to the cultures, implying that endogenous IL-10 induced by HHV-6 infection inhibited IL-12 production [43]. Selective suppression of IL-12 production induced by HHV-6 infection independently of viral replication was also reported [44,45]. Disharmony of cytokine production—that is, overproduction of IL-10 and suppression of IL-12—may result in impairment of the protective immune response against HHV-6. Interestingly, the relationship between IL-10 production by monocytes and dysfunction of monocyte-derived DCs has also been speculated upon in regard to human cytomegalovirus [46], which is a β-herpesvirus similar to HHV-6, suggesting that there are common mechanisms of IL-10 production by DCs in β-herpesvirus infections.

The various effects of HHV-6 on cellular functions have been clarified. However, these data were obtained from in vitro experimental systems; therefore, it is doubtful whether the same phenomena occur in vivo. Because HHV-6 infection is restricted to humans, no animal model is available. HHV-6 reactivation occurs frequently in patients with immunodeficiency diseases or conditions, such as hematopoietic stem cell transplantation, organ transplantation, and AIDS. However, the functions of monocytes and DCs are also affected by underlying diseases and immunosuppressive drugs; therefore, monocytes obtained from these immunodeficient patients do not seem suitable for examining the effect of HHV-6 infection in vivo. To overcome these problems, we used monocytes isolated from a patient with DIHS. The pathogenesis of this condition has recently been clarified. In patients with DIHS, severe HHV-6 reactivation is induced following drug allergy [47]. Because DIHS occurs in immunocompetent patients, the monocytes and lymphocytes of these patients seem to be useful for studying the biologic effects of HHV-6 infection on the immune system in vivo. Our data clearly showed that HHV-6-induced impairment of DC differentiation from monocytes certainly occurs in vivo.

In summary, we found that differentiation of myeloid DCs from monocytes is severely impaired by infection with either HHV-6A or HHV-6B. Our present data suggest a novel concept for the mechanism of HHV-6-induced immunodeficiency. In light of the evidence that HHV-6 reactivation occurs frequently in immunodeficient patients, clarification of the pathogenesis of HHV-6-induced immunodeficiency and restoration of the impaired immune system in HHV-6 infection seem important issues.

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Overexpression of SOCS3 inhibits astrogliogenesis and promotes maintenance of neural stem cells

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Abstract

To investigate the effects of suppressors of cytokine signaling 3 (SOCS3) on neural stem cell fate, stem cells were infected with an adenoviral vector expressing SOCS3. Three days later, western blot analysis and immunocytochemical analysis revealed that the protein level of MAP2 and the number of MAP2-positive cells were significantly increased in SOCS3-transfected cells, whereas the protein level of GFAP and the number of GFAP-positive cells were significantly decreased. Furthermore, promoter assay revealed a significant reduction in the transcriptional level of signal transducer and activator of transcription 3 (Stat3) in the transfected cells. In addition, the mRNA levels of Notch family member (notch1) and inhibitory basic helix-loop-helix (bHLH) factors (hes5 and id3) were significantly up-regulated 1 day after overexpression of SOCS3. Three days after transfection, the mRNA level of hes5 was significantly decreased, whereas that of notch1 was still up-regulated. Moreover, all of SOCS3-positive cells expressed Nestin protein but did not express MAP2 or GFAP proteins. These data indicate that overexpression of SOCS3 induced neurogenesis and inhibited astrogliogenesis in neural stem cells. Our data also show that SOCS3 promoted maintenance of neural stem cells.

Keywords: astrogliogenesis, neural stem cell, neurogenesis, suppressors of cytokine signaling 3.

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It is well known that Stat3 plays crucial roles in the determination of neural stem cell fate. Enhancement of Stat3 activity in neural stem cells leads to subsequent glial differentiation (Bonni *et al.* 1997; Rajan and McKay 1998). leukemia inhibitory factor (LIF) as well as ciliary neurotrophic factor (CNTF) and interleukin (IL)-6 can activate the Janus kinase (Jak)-signal transducer and activator of transcription (Stat) signaling pathway and promote astroglial differentiation (Johe *et al.* 1996; Bonni *et al.* 1997). Phosphorylated Stat3 associates with the transcriptional coactivators CREB binding protein (CBP/p300) to activate expression of astrocyte-specific genes (Nakashima *et al.* 1999). This gliogenic function is suppressed during the expression of pro-neural basic helix-loop-helix (bHLH) factors. Pro-neural bHLH factors are highly expressed during neurogenesis, and inhibit glial differentiation through sequestering Smad1-CBP/p300 away from glial-specific genes by inhibiting activation of Stat3 (Gangemi *et al.* 2004). In addition, DNA methylation of glial genes during the neurogenic period also inhibits glial differentiation by suppressing glial gene transcription and inhibiting Stat3 phosphorylation (Takizawa *et al.* 2001; Sun *et al.* 2003).

Stat can be phosphorylated by Jak, and can homo- or heterodimerize, and translocate to the nucleus, where it binds to specific consensus sequences to control various target genes (Levy and Lee 2002). Recently, many studies have shown that this Jak-Stat pathway is negatively regulated by suppressors of

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Abbreviations used: APRE, acute-phase response element; bHLH, basic helix-loop-helix; CIS, cytokine-inducible SH2 domain-containing protein; CNTF, ciliary neurotrophic factor; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein-5-isothiocyanate; IL-6, interleukin-6; Jak, Janus kinase; LIF, leukemia inhibitory factor; Lz, β-galactosidase; moi, multiplicity of infection; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; SOCS, suppressors of cytokine signaling; Stat, signal transducer and activator of transcription.

