表1 2005 年国勢調査に基づく MR3 期および 4 期接種対象者数の推定

【MR3 期】	全国（人）	回答市区町村（人）	回答市区町村/全国(%)
12 歳	1,187,516	1,076,656	90.66
13 歳	1,209,248	1,119,514	92.58
12 歳×0.25 ＋ 13 歳×0.75	1,203,815	1,108,800	92.11

【MR4 期】	全国（人）	回答市区町村（人）	回答市区町村/全国(%)
17 歳	1,313,462	1,190,515	90.64
18 歳	1,357,096	1,254,855	92.47
17 歳×0.25 ＋ 18 歳×0.75	1,346,188	1,238,770	92.02

表2 MR3 期および 4 期接種率算定値の比較

【MR3 期】	接種対象者数（人）		接種率[*3]
	全国	回答市区町村[*2]	(%)
厚生労働省公表数	1,191,047	1,097,073	86.4
2005 年国勢調査時 9 ～ 10 歳[*1]	1,199,374	1,104,743	85.8

[*1] 早生まれ（1 ～ 3 月生まれ）の 9 歳人口＋遅生まれ（4 ～ 12 月生まれ）の 10 歳人口
[*2] 全国接種対象者数 ×92.11%
[*3] MR3 期被接種者数（＝ 947,883）／回答市区町村における接種対象者推定数（＝ 1,104,743）

【MR4 期】	接種対象者数（人）		接種率[*6]
	全国	回答市区町村[*5]	(%)
厚生労働省公表数	1,224,040	1,126,362	78.2
2005 年国勢調査時 14 ～ 15 歳[*4]	1,225,724	1,127,911	78.1

[*4] 早生まれ（1 ～ 3 月生まれ）の 14 歳人口＋遅生まれ（4 ～ 12 月生まれ）の 15 歳人口
[*5] 全国接種対象者数 ×92.02%
[*6] MR4 期被接種者数（＝ 880,934）／回答市区町村における接種対象者推定数（＝ 1,127,911）

　一方，分母となる MR 3 期接種対象者数は，各市区町村の担当者の負担を考えて各自治体に問い合わせず，2005 年国勢調査に基づく年齢別人口[6,7]を利用して推定した．日本全体の年齢別四半期別人口は国勢調査結果から入手できるが，市区町村別年齢別四半期別人口は公表されていないため，全回答市区町村の MR 3 期接種対象者数（中学 1 年生人口）および MR 4 期接種対象者数（高校 3 年生年齢相当者人口）を下記の手順で推定した．

　まず，回答のあった各市区町村における国勢調査上の 12 歳人口を加算して全回答市区町村の国勢調査上の 12 歳人口を求め，同様に 13，17，18 歳の人口を求めた．次に，1 学年は 1/4 の早生まれと 3/4 の遅生まれから構成されているため，12 歳合計人口×0.25 と 13 歳合計人口×0.75 の合計を全回答市区町村の MR 3 期接種対象者数の仮推定数とし，同様にして全回答市区町村の 17 歳と 18 歳人口から MR 4 期接種対象者数の仮推定数を求めた．また，国勢調査による 12 歳および 13 歳人口を 1：3 の比率で加算した数を日本全体の MR 3 期接種対象者数とし，同様に 17 歳と 18 歳人口を 1：3 の比率で加算して全 MR 4 期接種対象者とした．

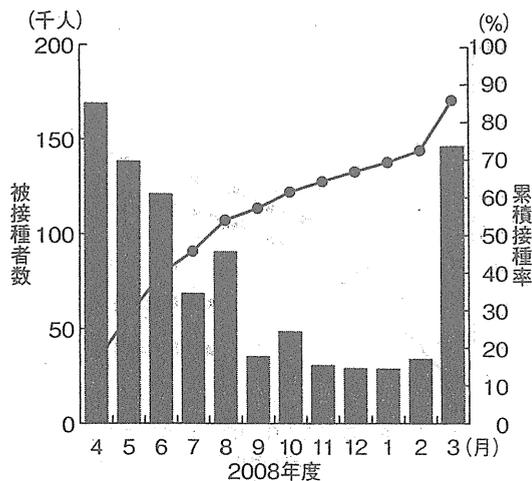　全国の接種対象者数と全回答市区町村の仮推定数を比較したところ，MR 3 期，4 期接種対象

図1 中学1年生における(MR3期)全国MRワクチン累積接種率
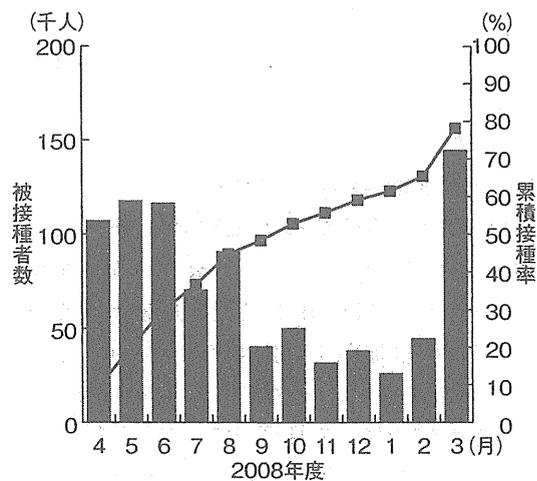
棒グラフは被接種者数,折れ線グラフは累積接種率を表す.

図2 高校3年生年齢相当者における(MR4期)全国MRワクチン累積接種率

棒グラフは被接種者数,折れ線グラフは累積接種率を表す.

者共に,後者は前者の約92%であった(表1).年齢別の人口差を考慮して,2005年国勢調査時の1～3月生まれの9歳人口と4～12月生まれの10歳人口の合計を2008年度の12～13歳の中学1年生人口(=全国MR3期接種対象者数)とし,同様に2005年の1～3月生まれの14歳人口と4～12月生まれの15歳人口の合計を2008年度の17～18歳の高校3年生年齢相当者の人口(=全国MR4期接種対象者数)としたうえで,これらの人口の92.11%ないし92.02%をそれぞれ全回答市区町村におけるMR3期,4期の接種対象者数の推定数とし,累積接種率算定の母数とした(表2).

倫理面への配慮:本研究は,すべての個人情報を排除して行ったため,倫理面で特段の問題はないと考える.

III. 結 果

1. 調査票の回収率および調査票によるMR3期被接種者総数

全国1,948か所の市区町村にアンケート用紙を郵送し,2008年4月～2009年3月の各月におけるMR3期および4期の接種者数を問い合わせた.2009年7月6日までに1,750か所の市区町村から回答が得られ,回収率は89.8%(1,750/1,948)となった.1,750か所の市区町村におけるMR3期被接種者数は合計94万7,883人,MR4期被接種者数は合計88万934人であった.

2. 月別MR3期および4期の被接種者数

回答を得た1,750市区町村において,MR3期接種を受けた中学1年生の数は2008年4月が16万9,391人,5月に13万8,683人,6月は12万1,773人と漸減,7月には6万9,332人まで減少した.8月には9万2,565人とやや持ち直したものの,9～11月は3～4万人台,12月と2009年1月には2.9万人台に減少し,3月に14万7,847人に増加した(図1).

一方,同じ市区町村において,MRワクチン4期接種を受けた高校3年生および同年齢相当者の数は,2008年4～6月を除いてMR3期と同様に経過した.すなわち,4月には10万7,292人,5,6月にはそれぞれ11万7,986人,11万6,813人と若干増加し,7月に7万746人に減少した後,8月に9万1,105人に持ち直した.しかし,9月は約4万人,10月は約5万人,

さらに11〜12月は3万人台に減少し，2009年1月には約2.6万人にまで減少した．2月には約4.5万人にまで回復し，3月には14万4,792人に急増した（図2）．

3．MR 3 期全国累積接種率

月別 MR 3 期被接種者累積数を分子とし，2005年国勢調査時の9〜10歳人口から推定した全回答市区町村 MR 3 期接種対象者数を分母として累積接種率を算定すると，2008年4月は15.3%，5月には27.7%，6月には38.9%，7月には45.2%，8月には53.5%と緩やかに上昇していたが，9月から2009年2月の間には累積接種率曲線の上昇が鈍化し，3月に急激に上昇して85.8%［95%信頼区間：85.7〜85.9%］に達した（図1）．

一方，同様にして MR 4 期の累積接種率を求めると，2008年4月は9.5%，5月には20.0%，6月には30.3%，7月には36.6%，8月には44.7% と MR 3 期の累積接種率よりも緩やかに上昇していた．9月から2009年2月の間はさらに上昇の程度が鈍くなったが，3月に急に上昇して78.1%［95%信頼区間：78.0〜78.2%］に達した（図2）．

4．MR 3 期と 4 期の累積接種率の比較

MR 3 期の累積接種率は MR 4 期の累積接種率よりも全体に高く推移していた．両者の差は2008年4月には約5.8%であったが，5月には7.7%，6月には8.6%と次第に広がり，8月には8.8%まで開いた．9〜11月は差が8.4〜8.5%であったが，12月に7.7%の差になり，2月には7.1%まで縮小したものの，2009年3月における最終的な差は7.7%であった（図3）．

5．厚生労働省公表接種率との比較

厚生労働省は，MR 3 期および 4 期の接種対象者数と被接種者数の報告を全国自治体に求めて，2008年12月31日現在における県別接種率を公表している[8,9]．これによれば，全国の接種対象者数は MR 3 期が119万1,047人，MR
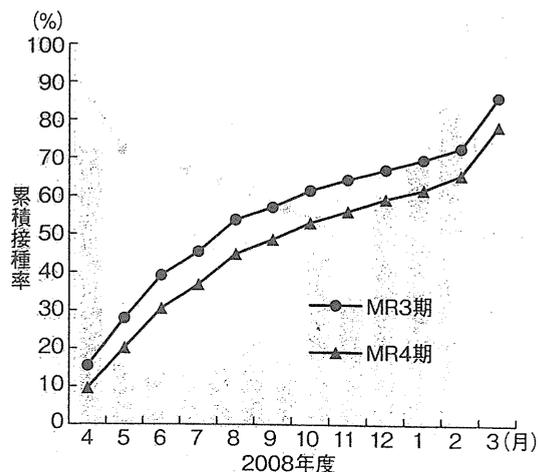

図3　MR ワクチン3期および4期累積接種率の比較

4 期が122万4,040人であり，麻疹単抗原ワクチンおよび風疹単抗原ワクチン被接種者を除いた，MR ワクチン被接種者のみの数は MR 3 期が78万7,069人，MR 4 期が70万9,602人となっており，この時点での MR ワクチンの接種率は MR 3 期が66.1%，MR 4 期が58.2%となる．

一方，われわれが求めた12月までの累積接種率は MR 3 期が66.7%，MR 4 期が59.0%であり，厚生労働省公表の接種率にきわめて近似している．また，われわれが回答を得た市区町村の接種対象者数を2005年国勢調査データを基に推定して求めた2009年3月の最終累積接種率は，MR 3 期が85.8%，MR 4 期が78.1%であった．一方，全回答市区町村の接種対象者を厚生労働省公表数から推定して求めた接種率は MR 3 期が86.4%，MR 4 期が78.2%となり，互いによく近似した値であった（表2）．

IV．考　察

2008年が MR 3 期および 4 期接種の初年度であったためか，MR 3 期，4 期共に累積接種率の伸びは悪く，2009年3月末までにそれぞれ85.8%，78.1%であった．今回の調査では累積接種率算定に当たり，各自治体での接種対象者

2096

数の合計ではなく，2005年国勢調査による全国9～10歳人口，および14～15歳人口を基に接種対象者数を推定し，これらを分母として用いた．算定に用いた推定数と実際の接種対象者数には多少の相違があると思われるが，厚生労働省が全国市区町村の対象者数を合計して公表した全国のMR3期および4期接種対象者数と今回用いた推定値との差は，MR3期対象者数では0.6%，MR4期対象者数では0.1%程度で，きわめて近似しており，算定された累積接種率は信頼できる値と考えられる．

今回の調査では，2008年4～6月の3か月間の被接種者数は10万人以上であったが，9月から2009年2月までは10月を除いて2～3万人台で推移し，年度末の3月に14万人台へと急激に増加していた．年度初めと年度末に被接種者数が多く，秋から冬には被接種者数が少ないという経過は，MR2期接種の場合にも観察されている[10]．

麻疹の感受性者と感受性者密度を下げて，麻疹の流行を防ぐためには，累積接種率が95%に達する必要がある．MR3期の累積接種率曲線とMR4期の累積接種率曲線の経過がほとんど同様であり，さらにはMR2期の累積接種率曲線の経過と類似している．これは，接種対象年齢が相違しても，接種を受ける側の動向が一様であるためと考えられる．したがって，MR2，3，4期の累積接種率は早期接種を勧める対策を講じればいずれも上昇することが期待できる．MR3期，4期の被接種者数が9月以降減少することを考慮すると，年度初めの学校行事の前にMR3期，4期接種を済ませることを目標とすることで累積接種率の向上が期待できる．

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Characterization of Neutralizing Epitopes of Varicella-Zoster Virus Glycoprotein H[▽]

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Varicella-zoster virus (VZV) glycoprotein H (gH) is the major neutralization target of VZV, and its neutralizing epitope is conformational. Ten neutralizing human monoclonal antibodies to gH were used to map the epitopes by immunohistochemical analysis and were categorized into seven epitope groups. The combinational neutralization efficacy of two epitope groups was not synergistic. Each epitope was partially or completely resistant to concanavalin A blocking of the glycomoiety of gH, and their antibodies inhibited the cell-to-cell spread of infection. The neutralization epitope comprised at least seven independent protein portions of gH that served as the target to inhibit cell-to-cell spread.

Varicella-zoster virus (VZV) glycoprotein H (gH) is the major target for neutralization (4, 5, 7, 9, 18), and it plays an important role in viral entry and cell-to-cell spread of infection (1, 3, 11, 12, 15). We isolated human monoclonal antibodies (MAb) to gH using an antibody library called AIMS4 constructed from B-lymphocyte-rich tissues of several dozen people (6, 19). Nine clones were selected for their neutralizing ability and their Fab sequences of heavy (H) and light (L) chains and used, in addition to TI-57, an anti-gH human MAb from a hybridoma, to characterize the neutralization epitopes of gH (18). Our system makes it possible to use the Fab form, which has about one-third the molecular weight of immunoglobulin G (IgG), in order to eliminate the spatial interaction between the Fc or other unreacted Fab of IgG molecules on one gH molecule. The neutralizing epitopes of gH are conformational, making gH hardly detectable by Western blot or enzyme-linked immunosorbent assay, and therefore, the conformational epitopes were mapped immunohistochemically. The combinational neutralizing activity between two species of Fab protein A (Fab-pp) forms and the inhibition of cell-to-cell infection were characterized, and the neutralization domain of gH was found to comprise a cluster of the seven neutralization epitopes and to prevent cell-to-cell infection.

Human embryonic lung cells were used to propagate Oka varicella vaccine, and cell-free virus was obtained by sonication of infected cells in SPGC medium (phosphate-buffered saline [PBS] containing 0.1% sodium glutamate, 5% sucrose, and 10% fetal bovine serum) followed by centrifugation (13, 14, 16).

Except for TI-57, each MAb was expressed in two forms: Fab-pp and Fab with an avidin tag (Fab-Avi-tag). Fab-pp corresponds to an Fab molecule fused with two domains of the Fc-binding protein A from *Staphylococcus aureus* (8) and purified on an IgG-conjugated column (19). Fab-Avi-tag is composed of an Fab bearing a 23-amino-acid-long peptide tag that can be biotinylated by the bacterial BirA biotin ligase (1). Fab-Avi-tag antibodies were purified by using SoftLink soft release avidin resin (Promega, Madison, WI).

To map the neutralizing epitope by Fab-pp, VZV-infected cells in 24-well plates were fixed by air-drying and then with 50% methanol and 50% acetone. The Fab-pp form (5 μg/ml in 0.5 ml of PBS with 3% skim milk) was used to block gH epitopes for 24 h at 4°C, and then 0.1 ml containing 1 to 10 μg Fab-Avi-tag was added and incubated at 4°C overnight. After incubation with streptavidin conjugated with peroxidase, competition for the gH epitope by the first Fab-pp and the challenging Fab-Avi-tag reaction was visualized by using a Dako liquid diaminobenzidine substrate chromogen detection system (17).

To assess the relationship between the glycomoiety and epitope, VZV-infected cells in eight-chamber culture slides were fixed by air drying and 50% methanol and 50% acetone. Then, the cells were treated with 0.5 ml/well of 200 μg/ml concanavalin A (ConA) (Wako Pure Chemical Industries Ltd., Osaka, Japan) in PBS for 1 h and with bovine serum for 1 h. After being washed with PBS, the cells were incubated with 1 μg/ml Fab-pp from each clone or 1:50-diluted zoster serum at 37°C for 1 h, washed with PBS, and incubated with fluorescein isothiocyanate (FITC)-conjugated anti-human IgG (H+L) rabbit serum (Wako) at 37°C for 1 h. The cells were observed under a fluorescence microscope.

The cells in six-well plates were infected with 50 PFU/0.05 ml of cell-free virus for 1 h and incubated for 1 h without antibody after washing the cells and then in the medium containing 500 μg/ml of the Fab-pp of clones 10, 11, 24, 36, 60, or 94 for 4 days without a change of medium (19). After fixation with 5% formalin, the cells were stained with methylene blue.

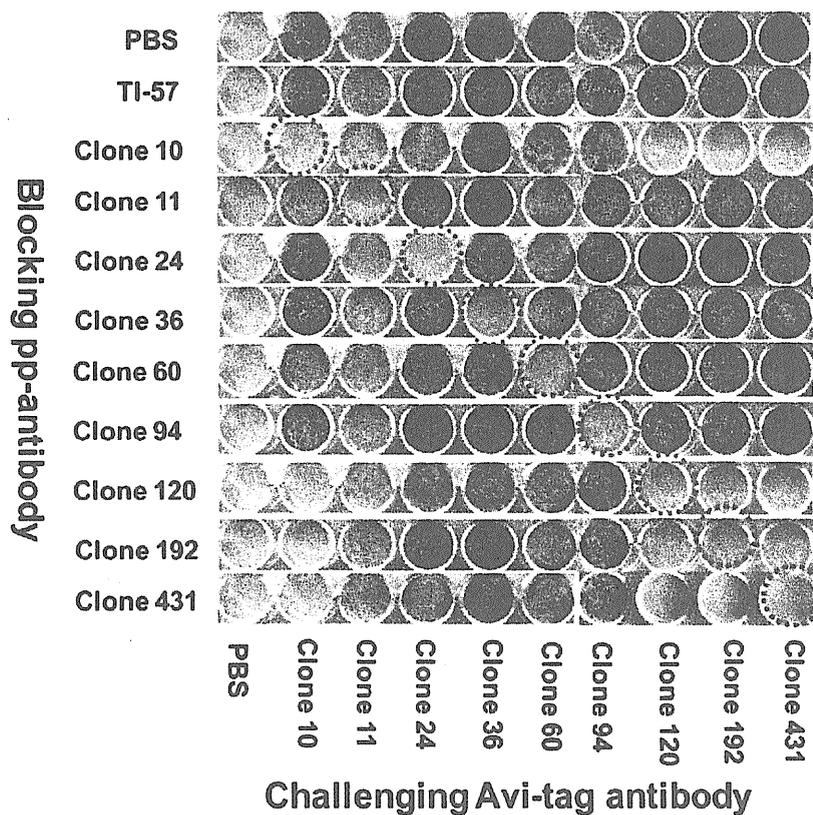Blocking with PBS failed to inhibit the staining with each

FIG. 1. Epitope mapping of gH by competitive immunostaining. Infected cells were first blocked by Fab-pp and then challenged by Fab-Avi-tag for visualization of the reaction of gH and Fab-Avi-tag. When PBS was used as the blocking agent, the challenging Fab-Avi-tag recognized the gH epitope, resulting in positive staining of infected cells, as shown in the top lane. When an Fab-pp successfully blocked the reaction with Fab-Avi-tag, the staining was blocked, as marked by dotted red circles around cultures with the homologous antibody combinations. When the Fab-pp and Fab-Avi-tag recognized different epitopes, infected cells were stained. Clones 10, 120, 192, and 431 showed identical reaction profiles, indicating that they belonged to the same epitope group. TI-57 blocking allowed staining by all kinds of Fab-Avi-tag, indicating that TI-57 recognized different epitopes than the other Fab-pp.

Avi-tag antibody, and all the infected cells were positively stained (Fig. 1). Blocking with a homologous Fab-pp blocked the immunostaining with Avi-tag antibody, as shown by the red circles. The Fab-pp of clones 11, 24, 36, 60, and 94 failed to block binding by the clone 10 Avi-tag antibody, and the Fab-pp of clones 120, 192, and 431 blocked binding by the clone 10 Avi-tag antibody, indicating that the epitope of clone 10 was similar to those of clones 120, 192, and 431 but different from those of clones 11, 24, 36, 60, and 94. The Fab-pp of clones 24, 36, 60, and 94 blocked only homologous combinations with each Avi-tag antibody and failed to block binding with the other Avi-tag antibodies. Altogether, epitope mapping of clones indicated the presence of six epitope groups, 10, 120, 192, and 431; 11; 24; 36; 60; and 94, in the neutralization domain of gH.

TI-57 antibody did not block any reaction of Avi-tag antibodies with clones 10, 120, 192, 431, 11, 24, 36, 60, or 94, even when used at 20 μg/ml to block the epitope of gH. This suggested that the epitope recognized by TI-57 was different from those recognized by the nine clones. TI-57 is not produced any more, and the amount of TI-57 was not sufficient to perform further work with TI-57. The target epitopes of gH for neu-

tralization were defined by the 7 groups of gH antibodies, 10, 120, 192, and 431; 11; 24; 36; 60; 94; and TI-57.

ConA presents as a tetramer with a molecular mass of approximately 108,000 Da, while the molecular masses of Fab-pp and gH are 50,000 and 120,000 Da, respectively. Tetrameric ConA efficiently inactivates viral infectivity (8) and may interfere with the interaction between Fab-pp and the epitope because of its spatial bulkiness when it reacts with the glycomoiety near the target epitope (20). Figure 2 shows the specificity of immunofluorescence and a comparison of the immunofluorescent staining by each antibody clone with and without ConA treatment. The intensity of staining by zoster serum was reduced by ConA treatment, possibly due to blocking of the interaction of the antibody with viral glycoproteins. Staining of infected cells by clone 36 with and without ConA and the contrast between infected and uninfected cells were not affected by ConA treatment, while those parameters in the other clones were reduced slightly or greatly by ConA treatment. Six epitopes were located near the glycomoiety of gH and not in the glycomoiety itself, and the epitope recognized by clone 36 was remote enough to evade spatial blocking by the tetrameric ConA bound to the glycomoiety. This indicated that the epitopes rec-
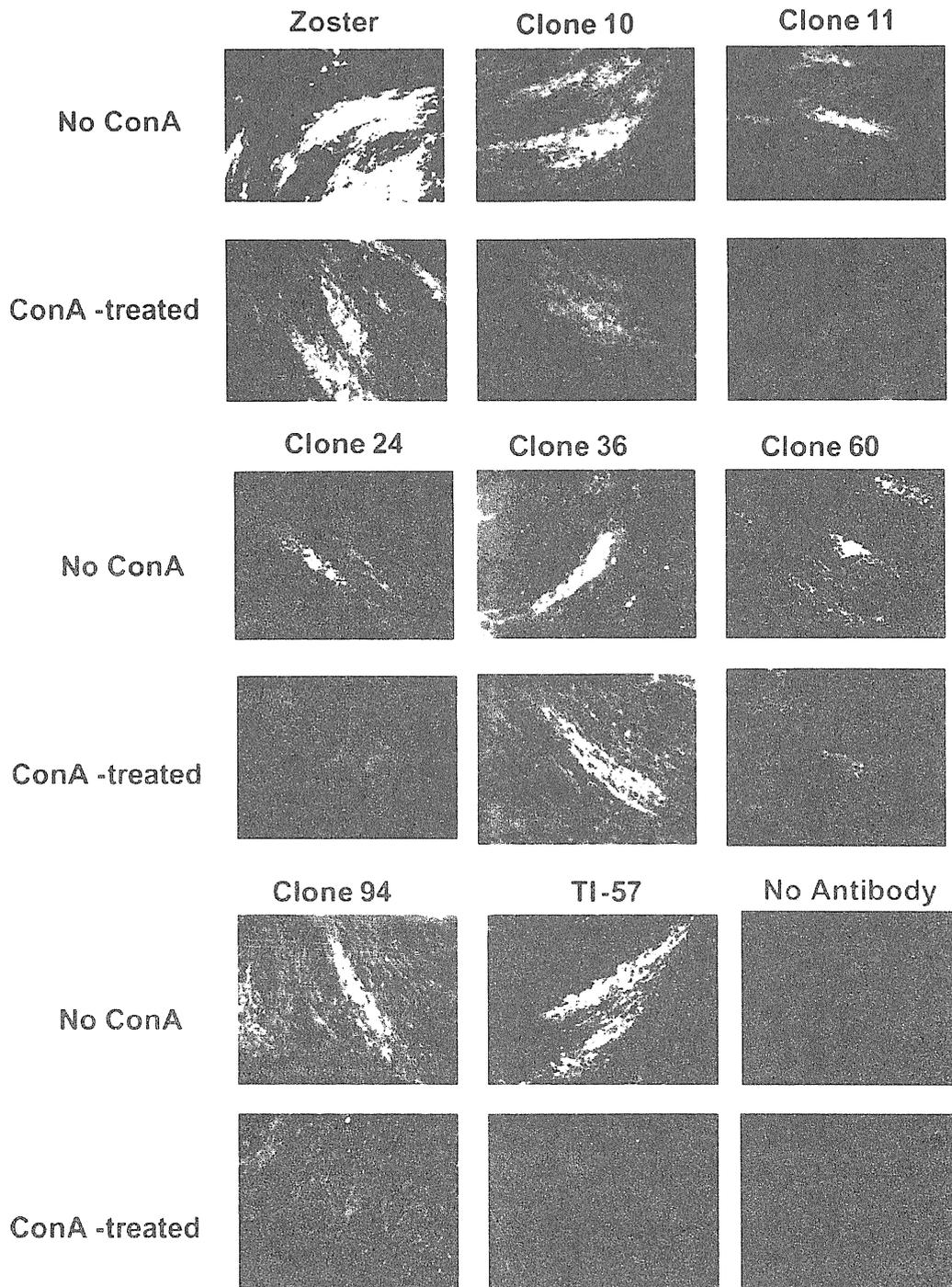
FIG. 2. Interference with epitope recognition by binding of ConA to gH glycomoieties. The cells on eight-chamber glass slides were treated with 0.5 ml/well of 200 μg/ml ConA or PBS and then with Fab-pp, followed by staining with FITC-labeled anti-human IgG (H+L) rabbit serum. "No Antibody" indicates that infected cells were directly stained with FITC-labeled anti-human IgG (H+L) rabbit serum to determine the specificity of anti-gH MAb. The FITC staining of infected cells without and with ConA treatment is shown. The specificity of FITC staining of infected cells among surrounding uninfected cells and the FITC staining contrast with and without ConA treatment illustrate the effects of ConA treatment on the interaction of anti-gH MAb with infected cells.

ognized by anti-gH neutralizing MAbs were protein portions or at least not glycomoieties of gH that interact with ConA.

Figure 3 shows the successful inhibition of plaque formation by the six clones representing six epitope groups. The infected culture without antibody showed extensive cytopathology, but the typical cytopathology did not develop in the infected cultures treated with each Fab-pp.

Table 1 shows the genetic characterization of the H chains of each clone. The variable H chain (VH) gene sequences from framework region 1 to framework region 3 of the seven anti-
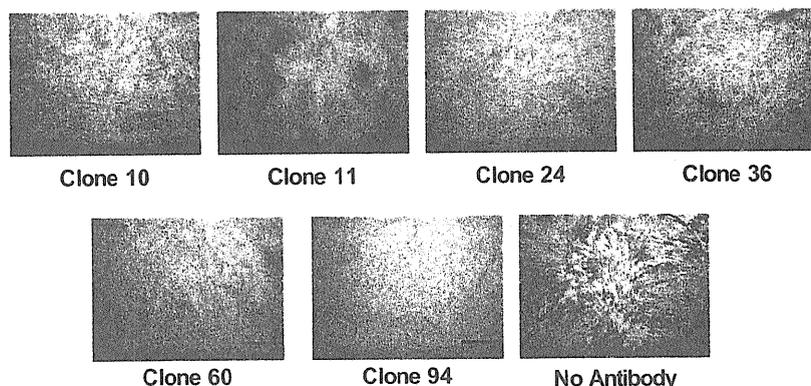
FIG. 3. Inhibition of cell-to-cell spread of infection and plaque formation by Fab-pp treatment. The cells were infected and incubated for 1 h without antibody and then treated with 500 μg/ml of Fab-pp of clone 10, 11, 24, 36, 60, or 94 for 4 days. The cells were fixed with formalin and stained with methylene blue. Plaques with extensive cytopathology were observed in infected cultures without antibody treatment, while treatment with 500 μg/ml of Fab-pp inhibited the spread of cytopathology. Bars indicate 1 mm.

body clones were compared with the germ line sequences listed in VBASE (VBASE Directory of Human V Gene Sequences; http://vbase.mrc-cpe.cam.ac.uk/). The original germ lines for clones 10 and 431, which recognize one epitope, were DP51 and DP50, respectively, and their complementarity-determining region 3 sequences were different in size and amino acids (19). Tyrosine-tyrosine in clone 10 and phenylalanine-tyrosine in clone 431 may form a hydrophobic core and recognize the same antigenic structure. Antibody clone 11, with no mutation in its VH gene, showed low neutralizing activity (50% inhibitory concentration, 8,000 nM), indicating a naïve antibody. The other clones had 18 to 29 nucleotide mutations in the VH genes, which may have contributed to efficient neutralizing activity by reacting with different epitopes.

Because these clones recognized multiple epitopes, we examined the combinational neutralizing activity of different Fab-pp forms by using a plaque reduction assay (14, 15, 19, 23). Eight kinds of Fab-pp clones were mixed in all possible combinations of two different clones, 36 in total, and the mean neutralizing efficacy was determined seven times. No combination of two clones reduced the number of plaques more than expected (data not shown), indicating that no combination of two clones had synergism. In a different virus system, the addition of MAbs to different functional domains, the V2, V3, or CD4 binding site of human immunodeficiency virus glycoprotein gp120, produced synergistic neutralization (21, 22). In

contrast, the multiple neutralizing epitopes of gH might have recognized one functional domain, resulting in no synergism.

Murine MAb 206 to gH neutralizes VZV and inhibits cell-to-cell fusion in gH+gL-transfected cells (2, 3). Prior reports indicate that gH can endocytose on its own, without gE (10), and that interaction with gE may lead to trans-Golgi network targeting. gE can increase endocytosis of gH lacking a YNKI endocytosis motif (11). The inhibitory mechanism of gH in the virus-to-cell or cell-to-cell interaction by neutralizing anti-gH MAbs is not clear. Some combinations of our MAbs that recognized six epitopes might be antagonistic, and further analysis of the relationship between neutralization and cell-to-cell infection among these MAbs might elucidate the gE-gH interaction in the virus-cell interaction and cell-to-cell infection.

All the anti-gH MAbs that had neutralizing activity against VZV blocked entry and egress of the viruses (19), suggesting that both infection by viruses and syncytium formation after infection would be mediated by the same single functional domain on the gH molecule (3–5, 7, 9, 11, 12, 15, 18). In conclusion, the neutralizing domain comprises at least seven independent protein portions of gH.

Nucleotide sequence accession numbers. The accession numbers of the H and L chains for clones 10, 24, 36, 60, 94, 120, 192, and 431 are AB063700 and AB064076, AB063703 and AB064219, AB063705 and AB064116, AB063707 and AB063990, AB063708 and AB064045, AB063700 and

TABLE 1. Genetic characterization of the variable H chains compared with germ line sequences in VBASE[a]

Clone	Germ line	No. of mutated nt/total no. of nt[b]	No. of mutated nt in:					Neutralization titer[c] (IC_{50} nM)
			FR1	CDR1	FR2	CDR2	FR3	
010	DP-51	24/291	9	6	2	1	6	40
011	DP-10	0/296	0	0	0	0	0	8,000
024	DP-35	29/296	3	5	4	9	8	3.0
036	DP-67	27/294	9	2	2	6	8	400
060	DP-49	28/294	3	3	4	11	7	25
094	DP-46	21/294	2	2	1	8	8	0.12
431	DP-50	18/296	1	2	1	10	4	200

[a] nt, nucleotides; FR; framework region, CDR; complementarity-determining region.
[b] The regions covered by the primers used for PCR were excluded.
[c] Neutralization titers are from Suzuki et al. (19). IC_{50}, 50% inhibitory concentration.

AB063929, AB063700 and AB063932, and AB355876 and AB355875, respectively.

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Relationship between U83 gene variation in human herpesvirus 6 and secretion of the U83 gene product

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Abstract The betaherpesvirus human herpesvirus 6 (HHV-6) has two variants. The U83 gene product of strain HST is a chemoattractant for monocytes. Here, we describe U83 gene variations that accumulated in variants A and B. A gene-variation hot spot was examined in 36 different strains and one donor DNA sample. U83 gene variations accumulated in variant A and in reactivated variant B after transplantation. None of the variant-A viruses encoded the signal peptide found in the B variant. U83 gene sequencing suggested that the variant A and B groups were separate, and that the variant B viruses could be further divided into the HST-Z29 type and another type with a shorter signal peptide. In a eukaryotic expression system, the HST-Z29 type of U83 gene product was secreted into the medium, a frame-shifted HST-Z29 type was partially secreted, and the variant-A type and a first-methionine knockout of the HST-Z29 type were not secreted.

Introduction

In 1986, human herpesvirus 6 (HHV-6) was isolated from patients with lymphoproliferative disorders [1]. Although the virus primarily replicates in CD4+ lymphocytes [2, 3], it can also be found as a latent infection in monocytes/macrophages and other cells of their lineage [4]. It is well established that HHV-6 causes exanthem subitum of infancy (ES) [5], but it is unclear if it causes any disease in adults. A number of different properties have led to the identification of two HHV-6 variants [6–13], which are called HHV-6A (variant A) and HHV-6B (variant B). The latter causes ES [14], and when latent, it can be reactivated in transplant recipients, who are immunosuppressed. It is unclear if variant A causes any disease. Previously, we sequenced the entire genome of an HHV-6B strain, HST, and found marked differences between some of its deduced protein sequences and those in HHV-6A [15].

The U83 gene of HHV-6B strain HST encodes a chemokine that functions as a chemoattractant for monocytes [16]. Similarly, the U83 gene of HHV-6A strain U1102 encodes a chemokine homolog, but it lacks a signal peptide sequence found in the B variant. Thus, U83 in strain U1102 is probably not secreted and therefore should not have chemotactic activity in vivo. In the present paper, to examine whether the differences in U83 between strains HST and U1102 were conserved among strains belonging to variants A and B, we determined the nucleotide sequences of the U83 genes in 36 HHV-6 isolates: 29 were HHV-6B, and 7 were HHV-6A. The isolates were obtained

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from individuals in Germany, Japan, Uganda, USA, and Zaire. In addition to confirming the differences between the U83 sequences of HHV-6 variants A and B, we found that while the deduced U83 sequence from HHV-6B ES patients was conserved, there were marked within-group differences among the reactivated HHV-6B isolates from transplant recipients.

Materials and methods

Cells and viruses

Umbilical cord blood mononuclear cells (CBMCs) were separated on a Ficoll-Conray gradient, cultured in RPMI 1640 medium containing 10% fetal calf serum (FCS), and stimulated with 5 µg/ml phytohemagglutinin for 2 or 3 days. The HHV-6 strain HST, an HHV-6B variant isolated from a patient with ES, was propagated in the fresh human CBMCs [5]. To prepare virus stocks, the virus was propagated in the fresh CBMCs stimulated with phytohemagglutinin [5]. When more than 80% of the cells showed cytopathic effects, the culture of infected cells was frozen, thawed twice, and spun at $1,500 \times g$ for 10 min, and the supernatant fraction was stored at $-80°C$ as a cell-free virus stock. To infect cells with virus, stimulated CBMCs (10^7 cells) were washed twice with phosphate-buffered saline (PBS), suspended in 1 ml of virus solution containing 10^7 50% tissue-culture infective doses per ml, and spun at $1,500 \times g$ for 40 min at 37°C to promote adsorption. To prepare viral DNA, infected cells were cultured for 2 or 3 days in RPMI1640 medium supplemented with 10% FCS and harvested when approximately 50% of the cells showed cytopathic effects. To analyze independently isolated viruses by PCR, CBMCs infected with the viruses were used.

The following viruses were kindly supplied by other researchers: strain Z29 by C. Lopez [17], strains GS and DA by Ablashi [6], strain U1102 by Honess [18], strain St. W. by Enders [19], and isolates CO1, CO7, and CO8 by Balachandran [6, 20]. Isolates AB69, AB84, BT344, BT348, BT425, BT449, BT451, BT499, BT519, BT552, BT562 (isolated in Fujita Health University Hospital by Yoshikawa et al.), and M2 [21] were obtained from patients after bone-marrow transplantation. Other HHV-6 viruses were isolated in our laboratory from patients with ES (isolated at Osaka University Hospital). Our isolates were passaged just a few times, only as necessary for isolation. All strains were confirmed to be HHV-6 by using specific monoclonal antibodies to HHV-6 (data not shown). We also obtained one DNA sample of variant A from the PBMCs of a healthy Japanese donor (No. 5628).

Preparation of HHV-6 DNA and PCR

To amplify the HHV-6 genomic DNA, it was isolated from virus-infected CBMCs with a QIAamp DNA blood mini kit (Qiagen), and 100 ng of total DNA was subjected to 30 cycles of PCR with Z-*Taq* polymerase (Takara Bio Inc., Otsu, Shiga, Japan). PCR for the DNA sequence analysis was carried out with the following primer pairs: U83-polmF (5′-AGCTGCGATACAGTCGTCAAGT-3′) and U83-polmR (5′-GATTATGGCAAACTATTAAACTG AC-3′), or U83-polmF2 (5′-AAGGTTAGTAATATGAG TGACATTACA-3′) and U83-polmR2 (5′-GATTATGGC AAACTATTAAACTGAC-3′). The PCR conditions for the DNA sequence analysis were as follows: 30 cycles of denaturation (98°C for 30 s), annealing (48°C for 30 s), and extension (72°C for 1 min) in a PCR Thermal Cycler TP480 (Takara Shuzo). The PCR products were cloned into pGEM-T Easy Vector (Promega) and sequenced. The PCR for protein expressability was carried out with the following primers: U83HST-kpnMet (5′-GGTACCACTT GTCGAAATGTTCATTTGGCT-3′), U83HST-kpnKOMet (5′-GGTACCACTTGTCGAAATATTCATTTGGCT-3′), U83GS-kpnMet (5′-GGTACCACTTGTCGAAATATCCA TTCGGCT-3′), and U83-bamTer (5′-GGATCCGATTCTT TGTCTAATTTCGACAATC-3′). The primer pair U83HST-kpnMet and U83-bamTer was used for the normal and frame-shifted products of strain HST. The primer pair U83HST-kpnKOMet and U83-bamTer was used for the first methionine knockout product of strain HST. The primer pair U83GS-kpnMet and U83-bamTer was used for the U83 product of strain GS. The PCR conditions for this assay were the same as above, except for the annealing temperature (55°C). The PCR products were cloned into pGEM-T Easy Vector (Promega) and confirmed by sequencing, and the confirmed inserts were then cloned into the Kpn I and BamH I sites of pEF-BOS-EGFP [22].

DNA sequencing and sequence analysis

Corresponding sequences were confirmed by direct sequencing of the PCR products amplified from DNA, with an ABI PRISM 3100 genetic analyzer (ABI). To assess the variation within a single strain or isolate, the PCR products were cloned into pGEM-T Easy Vector (Promega), following the manufacturer's instructions and at least 25 clones of each PCR product were sequenced. The obtained nucleotide sequence of the HHV-6 genome was analyzed to identify the potential open reading frame (ORF) using the software packages MacDNASIS ver. 3.7 (Hitachi Soft Engineering Co., Ltd., Yokohama, Japan) and ALIGN (Genome Information Research Center of Osaka University). Sequence comparisons were carried out using the

sequences of HHV-6A (U1102) [23], HHV-6B (Z29) [24], and HHV-6B (HST) [15].

The DDBJ accession numbers for the U83 genes of the isolates used in this paper are AB443457 to AB443531, and the number for the U83 gene of #5628 is AB465584.

Detection of U83 gene expression

HeLa cells transfected with GFP fusion plasmids were observed directly with a Radiance 2100 confocal laser scanning microscope. The amount of GFP fusion protein in the culture supernatant of HeLa cells transfected with the GFP fusion plasmids was measured with a Spectra MAX Gemini XS (Molecular Devices, Sunnyvale, CA). The mRNA of U83 in the transfected HeLa cells was extracted using an RNAqueous kit (Applied Biosystems Inc., Foster City, CA), and cDNA synthesis and real-time PCR were performed using a Power SYBR Green RNA-to-C_T 2-step kit (Applied Biosystems Inc.). Samples for real-time PCR were amplified for 40 cycles in a Perkin-Elmer model 7700 sequence detection system (Applied Biosystems Inc.) with continuous monitoring of the fluorescence under the following conditions: 2 min at 50°C and 10 min at 95°C, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. Each PCR run included one negative control and a dilution series of pGEM-U83HST (10^1–10^8 DNA copies/μl). The mRNA of β-actin in the transfected HeLa cells was assayed as described previously [25] using the cDNA described above. Each sample was analyzed in triplicate. Data were processed using the SDS 1.6 software package (Applied Biosystems Inc.).

The amplification of U83 cDNA by PCR was performed by the method described above using the primer pair of U83HST-kpnMet and U83-bamTer, or U83GS-kpnMet and U83-bamTer. The PCR products were purified by TA-cloning. The nucleotide sequence of U83 cDNA clones was determined using the DNA sequencing analysis procedure described above.

Results

U83 DNA sequence analysis

To examine whether the U83 deviation between strains U1102 and HST was conserved within variants A and B, we determined the nucleotide sequence of the U83 gene in 36 HHV-6 isolates classified as 7 variant A isolates and 29 variant B isolates. To avoid regional sequence biases, the isolates were obtained from individuals in Germany, Japan, Uganda, USA, and Zaire. The U83 genes of the HHV-6A strains, including strain U1102, encoded a chemokine homolog but did not contain a signal peptide sequence

(Table 1; Fig. 1a). The methionine (ATG) start site of the signal peptide that was encoded by variant B was changed in the entire variant A viruses except U1102 and #5628 to isoleucine (ATA) (Fig. 1b). Thus, variant A strains GS, DA, CO1, CO7, and CO8 lacked the upstream ATG shown in Fig. 1b. In the case of U1102 and #5628, the U83 chemokine was encoded in a different frame of the signal peptide sequence. Therefore, the variant A viruses we investigated did not encode a mature chemokine with a signal peptide. In variant B, the wild-type complete U83 gene was present in most of the viruses, even if it was a minor component (Table 1). The length of the signal peptide encoded in the U83 gene varied in different isolates and consisted of 19–21 amino acids, starting with the initial methionine (Fig. 1a). In HHV-6B strain HST, the polypeptide is cleaved between a glycine at position 20, which is downstream of the signal peptides, and a phenylalanine at position 21 [16]. Here, we found that 6 amino acids in this region, from the second methionine at position 17 to an isoleucine at position 22 of strain HST, were conserved in all of the HHV-6 strains we investigated (Fig. 1a). In the variant A viruses, except for U1102 and #5628, the C-terminal region of U83 was two amino acids shorter than in any of the variant B viruses (data not shown).

When HHV-6 was cultured for more than 50 generations, some mutations that changed the amino acid sequence of U83 accumulated. This occurred especially in strain HST, in which a nucleotide insertion disrupted the signal peptide of the U83 gene product. This mutation accumulated in more than 80% of the HST genomes by repeated viral passage in vitro (data not shown). Except for this change in vitro, however, we did not find that the viruses obtained from transplant recipients were obviously different from the ES viruses in terms of the proportion that possessed the signal peptide (see "ratio" in Table 1). Thus, whether or not viruses encoded a signal peptide might depend more on the virus type than on whether it was isolated from an acute infection or as a reactivated virus, or on the age and sex of the viral host. However, another mutation, in which a stop codon appeared in the middle of the U83 coding region, was more frequent in the reactivated viruses compared with the ES viruses (Table 1). These findings indicated that all of the HHV-6 genomes that caused ES encoded an active U83 gene product, and some of the U83s had a signal peptide. In contrast, two of the reactivated viruses, BT344 and BT449, never encoded a mature U83 protein (Table 1). In addition, the number of thymidines in the thymidine cluster of the signal peptide region was conserved in the variant A viruses but was variable in the variant B viruses (Table 1; Fig. 1b).

To assess the variation within an individual strain or isolate, we sequenced at least 25 clones of each investigated virus. A major sequence was found to represent more

Springer

— 126 —

Table 1 Comparison of U83 nucleotide sequences among HHV-6 strains

Strain	Subtype	Diagnosis	Country	Passage no.[e]	Similarity (%)[d]	Length (bp)	ORF[b]	Identity (%)[d]	Length (AA)	−5	−4	−3	−2	−1	0	1	2	3	4	Stop codon (%)	Mature ratio	Protein (%)[c]
Z29	B	AIDS	Zaire	?	100.0	0	Y	100.0	113					10.7	**75.0**	10.7	3.6				75.0	75.0
U1102	A	AIDS	Uganda	?	92.4	−2	N	87.6	97				100.0								0.0	
GS	A	AIDS	US	?	92.1		N	87.4	95	No met upstream of the signal peptide											0.0	
DA	A	CFS	Germany	?	92.1		N	87.4	95	No met upstream of the signal peptide											0.0	
CO1	A	Collagen vascular disease	Germany	?	92.1		N	87.4	95	No met upstream of the signal peptide											0.0	0.0
CO7	A	Collagen vascular disease	Germany	?	91.8		N	86.3	95	No met upstream of the signal peptide											0.0	
CO8	A	Collagen vascular disease	Germany	?	91.5		N	86.3	95	No met upstream of the signal peptide											0.0	
#5628	A	Healthy donor	Japan	0	92.5	−1	N	85.6	97					100.0							0.0	
AB69	B	Reactivation after BMTP	Japan	3	98.4	−3	Y	96.5	112			**96.6**	3.4								96.6	
AB84	B	Reactivation after BMTP	Japan	3	99.6	1	N	100.0	97						**16.1**	80.6	3.2			9.7	16.1	
BT344	B	Reactivation after BMTP	Japan	3	98.0	−2	N	97.9	97				92.0	8.0							0.0	
BT348	B	Reactivation after BMTP	Japan	3	99.2	1	N	100.0	97						**3.1**	90.6	6.3				3.1	
BT415	B	Reactivation after BMTP	Japan	3	98.0	1	N	100.0	97						**11.5**	80.8	7.7				11.5	
BT449	B	Reactivation after BMTP	Japan	3	98.0	−3	NS	100.0	50			**92.0**	8.0							100.0	0.0	35.6
BT451	B	Reactivation after BMTP	Japan	3	99.8	1	N	·100.0	97						10.7	75.0	10.7	**3.6**		7.1	14.3	
BT499	B	Reactivation after BMTP	Japan	3	99.2	2	N	100.0	97						3.7	14.8	69.0	**7.4**		7.4	11.1	
BT519	B	Reactivation after BMTP	Japan	3	98.2	−3	Y	96.5	112		3.4	**96.6**								13.8	82.8	
BT552	B	Reactivation after BMTP	Japan	3	99.4	3	Y	99.1	114							10.3	6.9	**69.0**	13.8	6.9	62.1	
BT562	B	Reactivation after BMTP	Japan	3	100.0	0	Y	100.0	113					3.8	**96.2**						96.2	
M2	B	Reactivation after BMTP	Japan	2	99.8	1	N	100.0	97					3.2	**19.4**	45.2	16.1	**3.2**	3.2	3.2	22.9	
HST	B	ES	Japan	2	100.0	0	Y	100.0	113						**84.0**	16.0					84.0	
St. W.	B	ES	Germany	?	97.1	−3	Y	96.5	112			**94.7**	5.3							5.3	89.5	

Strain	Subtype	Diagnosis	Country	Passage no.[e]	Similarity (%)[d]	Length (bp)	ORF[b]	Identity (%)[d]	Length (AA)	Comment[a]										Stop codon (%)	Mature ratio	Protein (%)[c]
										−5	−4	−3	−2	−1	0	1	2	3	4			
ES-1	B	ES	Japan	2	99.6	1	N	99.0	97						**12.0**	84.0	4.0				12.0	
ES-3	B	ES	Japan	2	99.8	1	N	100.0	97						**11.5**	84.6	3.8				11.5	
ES-5	B	ES	Japan	2	99.6	1	N	99.0	97						**20.0**	76.0	4.0				20.0	
ES-6	B	ES	Japan	2	99.8	1	N	100.0	97						**4.0**	88.0	8.0				4.0	
ES-7	B	ES	Japan	2	99.6	1	N	100.0	97						**7.7**	92.3					7.7	
ES-8	B	ES	Japan	2	99.6	2	N	100.0	97							25.9	70.4	**3.7**			3.7	
ES-11	B	ES	Japan	2	97.6	−3	Y	96.5	112	3.2	6.5	**90.3**									90.3	41.2
ES-12	B	ES	Japan	2	100.0	0	Y	100.0	113					4.0	**96.0**						96.0	
ES-13	B	ES	Japan	2	99.4	1	N	99.0	97						**8.0**	84.0	8.0				8.0	
ES-14	B	ES	Japan	2	99.8	1	N	100.0	97						**11.1**	81.5	7.4				11.1	
ES-16	B	ES	Japan	2	99.8	1	N	100.0	97						**20.0**	80.0					20.0	
ES-17	B	ES	Japan	2	100.0	0	Y	100.0	113					7.4	**88.9**	3.7					88.9	
ES-18	B	ES	Japan	2	99.8	1	N	100.0	97					4.0	**24.0**	64.0	8.0				24.0	
ES-21	B	ES	Japan	2	95.3	−2	N	97.9	97			**7.1**	89.3	3.6							7.1	
ES-24	B	ES	Japan	2	99.8	0	Y	100.0	113					10.3	**86.2**	3.7				86.2	

The numbers in the table show the percentage of the total sequenced clones obtained by TA cloning. The clones given in *bold* encoded a U83 gene product with a signal peptide and a functional chemokine

[a] The predominant nucleotide sequence of the signal sequence was compared to that of the HST signal peptide: the same length is shown as 0, if longer, the difference is shown as a positive number, and if shorter, as a negative number

[b] For the ORF, *Y* indicates that the dominant ORF encoded a U83 gene product with a signal peptide and functional chemokine, *N* indicates a U83 product without a signal peptide, and *NS* indicates a U83 gene product that did not encode a functional chemokine

[c] Mature protein ratio refers to the proportion of U83 gene product that had a signal peptide and functional chemokine, with the predominant nucleotide sequence of strain HST

[d] *Similarity* is the similarity of the DNA sequence to that of strain HST, *identity* is identity of the amino acid sequence to that of strain HST

[e] Passage number is the number of the sample for sequencing that is for virus isolation in each Japanese sample. The sample DNA of passage number *0* was obtained directly from PBMCs. As the passage numbers of the non-Japanese samples were not known, the passage numbers are shown as *?*

Fig. 1 a Amino acid sequence alignment of the U83 chemokine signal peptides of HHV-6 variants *A* and *B*. First and second methionines are *underlined*. The major and minor sequences are shown in *capital* and *small* letters, respectively. The *arrow* shows the cleavage site of the U83 signal peptide. The *gray* background indicates amino acids that are different from those of strain HST.
b Nucleotide sequence alignment corresponding to the U83 chemokine signal peptides of *A*. First and second ATG sites (methionine sites) are *underlined*. Only the major sequences are shown. A *gray* background indicates nucleotides that are different from those of strain HST

A

		Variant
HST.aa	MFIWLFIV-FFYAAYIGMAIGFIG	B
Z29.aa	MFIWLFIV-FFYAAYIGMAIGFIG	B
U1102.aa	MAIGFIC	A
GS.aa	MAIGFIC	A
DA.aa	MAIGFIC	A
CO1.aa	MAIGFIC	A
CO7.aa	MAIGFIC	A
CO8.aa	MAIGFIC	A
#5628.aa	MAIGFIC	A
AB69.aa	MFIWLFI--FFYAAYLGMAIGFIG	B
AB84.aa	mfiwlfiv-ffyaayigMAIGFIG	B
BT344.aa	MAIGFIG	B
BT348.aa	mfiwlfiv-ffyaayigMAIGFIG	B
BT415.aa	mfiwlfiv-ffyaayigMAIGFIG	B
BT449.aa	MFIWLFI--FFYAAYLGMAIGFIG	B
BT451.aa	mfiwlfiv-ffyaayigMAIGFIG	B
BT499.aa	mfiwlfivfffyaayigMAIGFIG	B
BT519.aa	MFIWLFI--FFYAAYLGMAIGFIG	B
BT552.aa	MFIWLFIVFFFYAAYIGMAIGFIG	B
BT562.aa	MFIWLFIV-FFYAAYIGMAIGFIG	B
M2.aa	mfiwlfiv-ffyaayigMAIGFIG	B
St.W.aa	MFIWLFI--FFYAAYLGMAIGFIG	B
ES-1.aa	mfiwlfiv-ffyaayigMAIGFIG	B
ES-3.aa	mfiwlfiv-ffyaayigMAIGFIG	B
ES-5.aa	mfiwlfiv-ffyaayigMAIGFIG	B
ES-6.aa	mfiwlfiv-ffyaayigMAIGFIG	B
ES-7.aa	mfiwlfiv-ffyaayigMAIGFIG	B
ES-8.aa	mfiwlfivfffyaayigMAIGFIG	B
ES-11.aa	MFIWLFI--FFYAAYLGMAIGFIG	B
ES-12.aa	MFIWLFIV-FFYAAYIGMAIGFIG	B
ES-13.aa	mfiwlfiv-ffyaayigMAIGFIG	B
ES-14.aa	mfiwlfiv-ffyaayigMAIGFIG	B
ES-16.aa	mfiwlfiv-ffyaayigMAIGFIG	B
ES-17.aa	MFIWLFIV-FFYAAYIGMAIGFIG	B
ES-18.aa	mfiwlfiv-ffyaayigMAIGFIG	B
ES-21.aa	mfiwlfi--ffyaaylgMAIGFIG	B
ES-24.aa	MFIWLFIV-FFYAAYIGMAIGFIG	B

B

HST.dna	ATGTTCATTTGGCTTTTTATTG---TTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
Z29.dna	ATGTTCATTTGGCTTTTTATTG---TTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
U1102.dna	ATGTCCATTCGGCTTTTTATTG-----GTTTTTTATACGGCATATATTGGTATGGCTATCGGATTTATATGT
GS.dna	ATATCCATTCGGCTTTTTATT----TGTTTTTTTATACGGCATATATTGGTATGGCTATCGGATTTATATGT
DA.dna	ATATCCATTCGGCTTTTTATT----TGTTTTTTTATACGGCATATATTGGTATGGCTATCGGATTTATATGT
CO1.dna	ATATCCATTCGGCTTTTTATT----TGTTTTTTTATACGGCATATATTGGTATGGCTATCGGATTTATATGT
CO7.dna	ATATCCATTCGGCTTTTTATT----TGTTTTTTTATACGGCATATATTGGTATGGCTATCGGATTTATATGT
CO8.dna	ATATCCATTCGGCTTTTTATT----TGTTTTTTTATACGGCATATATTGGTATGGCTATCGGATTTATATGT
#5628.dna	ATGTCCATTCGGCTTTTTATTG----GTTTTTTTATACGGCATATATTGGTATGGCTATCGGATTTATATGT
AB69.dna	ATGTTCATTTGGCTTTTTATT------TTTTTTTATGCGGCATATCTTGGTATGGCTATCGGATTCATCGGT
AB84.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
BT344.dna	ATGTTCATTTGGCTTTTTATT-----TTTTTTTTATGCGGCATATCTTGGTATGGCTATCGGATTCATCGGT
BT348.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
BT415.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
BT449.dna	ATGTTCATTTGGCTTTTTATT------TTTTTTTATGCGGCATATCTTGGTATGGCTATCGGATTCATCGGT
BT451.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
BT499.dna	ATGTTCATTTGGCTTTTTATTG-TTTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
BT519.dna	ATGTTCATTTGGCTTTTTATT------TTTTTTTATGCGGCATATCTTGGTATGGCTATCGGATTCATCGGT
BT552.dna	ATGTTCATTTGGCTTTTTATTGTTTTTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
BT562.dna	ATGTTCATTTGGCTTTTTATTG---TTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
M2.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
St.W.dna	ATGTTCATTTGGCTTTTTATT------TTTTTTTATGCGGCATATCTTGGTATGGCTATCGGATTCATTGGT
ES-1.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-3.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-5.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-6.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-7.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-8.dna	ATGTTCATTTGGCTTTTTATTG-TTTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-11.dna	ATGTTCATTTGGCTTTTTATT------TTTTTTTATGCGGCATATCTTGGTATGGCTATCGGATTCATCGGT
ES-12.dna	ATGTTCATTTGGCTTTTTATTG---TTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-13.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-14.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-16.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-17.dna	ATGTTCATTTGGCTTTTTATTG---TTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-18.dna	ATGTTCATTTGGCTTTTTATTG--TTTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT
ES-21.dna	ATGTTCATTTGGCTTTTTATT-----TTTTTTTTATGCGGCATATCTTGGTATGGCTATCGGATTCATCGGT
ES-24.dna	ATGTTCATTTGGCTTTTTATTG---TTTTTTTTTATGCGGCATATATTGGTATGGCTATCGGATTTATCGGT

than 70% of the U83 clones for most isolates (with the exception of five reactivated viruses and one ES-causing isolate), while clones producing each minor sequence represented less than 30% of each isolate (Table 2). Of the isolates for which less than 70% of clones encoded the major sequence, 60–70% of the clones from four reactivated viruses and the isolate that caused ES did so. Although a 50–50% representation of two different sequences was never found, clones of the reactivated M2

Table 2 Proportion of U83 clones containing a particular nucleotide sequence in each HHV-6 isolate

Virus	Major (%)[a]	Minor 1 (%)	Minor 2 (%)	Minor 3 (%)
HST	84	16		
Z29	75	11	11	
U1102	100			
GS	100			
DA	89	8		
CO1	91			
CO7	100			
CO8	70	30		
#5628	100			
AB69	97			
AB84	65	5		
BT344	88	8		
BT348	88	6		
BT415	81	12	8	
BT449	92	8		
BT451	68	11	11	
BT499	63	15	7	
BT519	83			
BT552	62	14	10	7
BT562	96			
M2	39	19	16	6
St.W.	89	5		
ES-1	84	12		
ES-3	85	12		
ES-5	76	20		
ES-6	84	8		
ES-7	88	8		
ES-8	70	22		
ES-11	90	6		
ES-12	96			
ES-13	84	8	8	
ES-14	81	11	7	
ES-16	76	20		
ES-17	89	7		
ES-18	64	24	8	
ES-21	86	7		
ES-24	86	10		

[a] Major means the predominant sequence

isolate in particular showed a marked variation in their U83 gene sequences.

In the short-type U83 signal peptide (valine at position 8 deleted), seen in isolates AB69, BT449, BT519, ES-11, and strain St.W., the isoleucine that is located 15 amino acids downstream of the first methionine in strain HST was changed to leucine. In contrast, in the long-type U83 signal peptide (bearing an additional phenylalanine) seen in isolate BT552, the isoleucine was conserved (Fig. 1a). In the short type, the nucleotides at HST positions 43 and 63, adenine and thymidine, respectively, of some BT344 and ES-21 clones were both changed to cytidines (Fig. 1b). The nucleotide change at position 43 caused an amino acid substitution, but the one at position 63 did not. However, most of the clones of these isolates did not encode the U83 signal peptide. The deduced amino acid sequences of these isolates showed that the addition of a thymidine to the thymidine-rich region was responsible for the one-amino-acid-shorter signal sequence type. There were no other nucleotide or amino acid changes in the signal sequence region of variant B, except as described above (Fig. 1).

In the short-type U83 signal peptide group, except for BT449, the leucine and methionine at positions 23 and 71 of the functional chemokine of strain HST were changed to serine and threonine, respectively. In all of the investigated sequences of BT449, a tryptophan at position 32 of the functional chemokine was changed to a stop codon. In the case of the variant A viruses, all of the investigated viruses had the same amino acid sequence, except for CO7 and CO8, which had a threonine at position 61 of the functional chemokine. In the case of the sample from the healthy Japanese donor (#5628), the amino acid sequence of U83 was the same as that of U1102, except for an isoleucine, a serine, a glycine, and a serine at positions 17, 50, 65, and 79, respectively, of the functional chemokine.

U83 gene expression

We next sought to determine whether functional differences in U83 were related to the differences in the DNA sequences reported above. Wild-type (WT), frame-shift-type (FS), and first-methionine knockout (KO) U83 gene products of strain HST and the U83 product of strain GS (GS) were expressed in HeLa cells. By observing cells expressing GFP-fusion versions of these proteins, we found that all of the U83 gene products were localized to the cytosol. Although the amounts of WT mRNA in the transfected cells was the lowest, and the amounts used for the FS, KO, and GS mutations were about 1.4, 2.9, and 2.0 times greater, respectively (Fig. 2a) than for WT, the amount of WT fusion protein in the transfected cells was lower than that of any of the other proteins (FS, KO, GS), the FS and KO fusion proteins were expressed at almost the
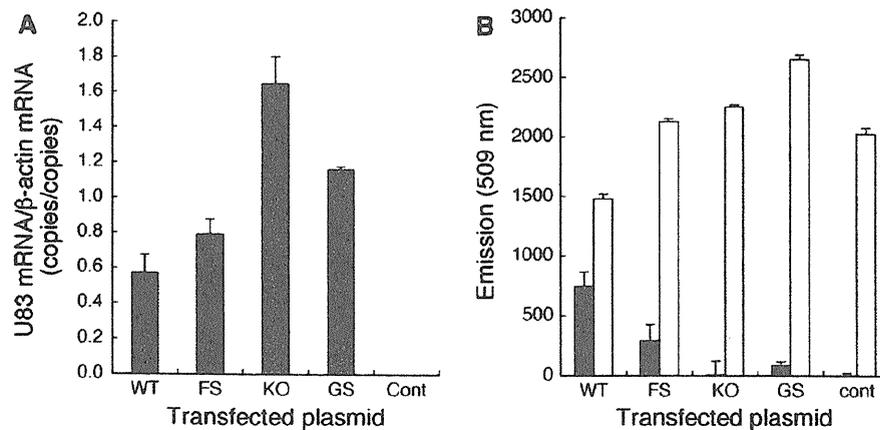
Fig. 2 U83 gene expression and product secretion from transfected cells. **a** U83 mRNA levels in transfected cells. The amounts of U83 and β-actin mRNAs in the cells were estimated by real-time RT-PCR. A housekeeping gene, β-actin, was used as a control. U83-GFP expression is normalized to β-actin expression. Each *bar* indicates the

average of triplicate samples. **b** Amount of GFP in the culture supernatant and cells. *Gray* and *white* bars indicate the fluorescence (excitation at 490 nm, emission at 509 nm) in the culture supernatant and cell lysate, respectively. Each *bar* indicates the average of four samples

same level, and GS was expressed at the highest level (Fig. 2b). However, the amount of fusion protein that was secreted differed from the expression levels for all four proteins (Fig. 2b). The WT U83 gene product showed the highest level of secretion into the medium, the FS U83 gene product was detected at lower levels, and the KO and GS U83 gene products were scarcely detectable in the medium. These results indicate that the U83 gene product of strain HST was secreted from the infected cells, but that of strain GS, which did not have a methionine site in its signal peptide region, was not secreted and remained in the cytosol, as would be expected.

U83 cDNA sequence analysis

We sought to determine whether the variation of U83 mRNA was generated in HeLa cells in which the WT, FS,

Table 3 Comparison of U83 mRNA nucleotide sequences in HeLa cells transfected with the U83 gene

Transfected genes	Length of signal sequence[a]			
	+2	+1	0	−1
WT			87	13
FS	14	77	9	
GS	No change of the nucleotide sequence of GS DNA			

The numbers in the table show the percentage of the total sequenced clones obtained by TA cloning. The clones on the **bold** encoded a U83 gene product with a signal peptide and a functional chemokine

[a] The nucleotide sequence of the signal sequence was compared to that of the HST signal peptide: the same length is shown as 0, if longer, the difference is shown as a positive number, and if shorter, as a negative number

and GS U83 gene products were expressed as described above. In observing cells expressing GFP-fusion versions of these proteins, although the variation of U83 mRNA in GS U83-gene-transfected cells was not found, there were variations of U83 mRNA in WT and FS U83-gene-transfected cells (Table 3). The variation was caused by a nucleotide insertion or deletion into the thymidine cluster of the signal sequence region. The variation of mRNA was the same as that of viral DNA. The data suggested that in-frame U83 mRNA was partially produced from frame-shifted U83 expression plasmid (Table 3).

Discussion

Here, we examined variations in the U83 gene sequence among strains and isolates of HHV-6 from different host sources. Our findings indicated that a high number of passages of HHV-6 viruses caused mutations in U83, which was particularly marked in the HHV-6B isolates, which accumulated single-nucleotide insertions or deletions in the thymidine cluster of the signal peptide region that determined the presence or absence of the U83 signal peptide. We therefore used fresh clinical isolates for nucleotide sequencing. U83 sequencing suggested that the variant A group was evolutionarily divergent from the B group and that the variant B viruses could be further separated into two subgroups, an HST-Z29 type and a short-U83 signal peptide type. Variation was also found among the clones of individual strains or isolates. Although the number of thymidines in the thymidine cluster in the signal peptide region differed (Table 1), a major U83 sequence was encoded by more than 70% of the clones of each

isolate (except for five reactivated viruses and one isolate that caused ES), and the minor sequences were encoded by less than 30% of the clones of each isolate (Table 2). The reactivated viruses tended to show more U83 sequence variation than the others, and the M2 isolate exhibited an especially marked variation in sequences. This may mean that U83 is dispensable for viral reactivation in patients who are immunosuppressed for a long period. In contrast, the major sequences of the essential genes U38 and U69 of the M2 isolate are dominantly expressed [21]. It is reasonable that we did not find any two sequences encoded by a 50% clonal frequency in an isolate, because the human body subjects the viruses to selective pressure, e.g., immunological pressure and translational efficiency, etc. In the case of the variant A viruses, the U83 DNA sequence was highly conserved. In particular, the signal peptide sequence was well conserved in these isolates, although two (U1102 and #5628) had a frame-shifted methionine site, and the others had no methionine upstream of the signal peptide. The conservation of the signal peptide DNA sequence in the variant A viruses may reveal a difference in selective pressure between the variant types, because this sequence varied slightly among the variant B viruses. The variation in the number of thymidines encoded in the sequence for the thymidine cluster of the signal peptide in the variant B viruses should lead to the translation of different products (Table 1). If translation from the U83 gene that had a frame-shifted methionine site did initiate with the actual first ATG in the variant B isolates, it would run off track within the sequences encoding the signal peptide and not produce any U83 product. As another possibility, we must also consider that translation might initiate with the ATG site of the functional U83 chemokine. However, if the sequence encoding the U83 signal peptide of the variant B viruses were easily misread during transcription, as shown in Table 3, U83 chemokines possessing the signal peptide might be translated nonetheless from the frame-shifted U83 genes. Further studies exploring the fluctuation of thymidine number in the thymidine cluster of the U83 signal peptide region on DNA replication and transcription in the variant B viruses may provide new insights into the molecular mechanisms of evolution and adaptation of this gene.

Although U83 is a dispensable gene, at least in vitro, the U83 divergence might contribute to cell tropism or pathogenic differences between the two variants in vivo, or between viruses in acute and latent infections. U83 of HHV-6B strain HST encodes a chemokine that functions as a chemoattractant for monocytes [16]. Similarly, the U83 genes of sequenced HHV-6A, as shown in Table 1, encoded a chemokine homolog, and the HHV-6A gene product expressed by E. coli functions as a chemokine [26]. However, none of the U83 genes of HHV-6A appeared to

contain a signal peptide sequence in vivo. Thus, the U83 gene product of variant A may not be secreted and therefore would not be expected to exert chemotactic activity in virus-infected cells, even though the gene product itself can function as a chemokine [26]. Our results in HeLa cells (Fig. 2) suggested that the U83 gene product of strain HST (WT) was secreted from infected cells, but the U83 KO gene product of strain HST and the U83 gene product of strain GS were not secreted and remained in the cytosol. A small amount of the U83 FS gene product of strain HST was secreted, but much of it remained in the cytosol. The secretion of a portion of the U83 FS gene product might have been the result of in-frame mRNA transcription by misreading of the thymidine cluster as shown in Table 3. But U83-GFP was not detected in culture supernatant using MT-4 cells transfected with the GFP-U83 constructs. Further, U83-GFP was not detected in culture supernatant using MT-4 cells transfected with the GFP-U83 constructs followed by HHV-6B (HST strain) infection. As MT-4 cells (T-cell line) might have a lot of receptors for U83-chemokine, and secreted U83 might be quickly trapped by MT-4 cells, we could not detect free GFP-U83 in culture supernatant. Our notion may be a suitable prediction about the secretion of U83 gene products; although we do not deny the potential that some viral proteins make a complex with U83 for secretion.

Although the U83 chemokine and its spliced isoforms can perform their expected biological functions, including inhibition of HIV-1 infection, leukocyte chemotaxis, and receptor internalization, when synthesized by E. coli [27, 28], non-secreted U83 gene products, like those of variant A viruses, should not be able to perform these functions in vivo. In the case of receptor internalization in HHV-6-infected cells, the U83 chemokine or its spliced isoform must be secreted into the cisternal space of the endoplasmic reticulum. Since the signal peptide is required for this secretion, the U83 gene products of variant A would not be functional for receptor internalization in HHV-6-infected cells in vivo. If U83 functions as a chemoattractant in vivo, it is possible that it facilitates variant B spreading by cell-to-cell infection, even in the presence of neutralizing antibodies, in contrast to variant A, which spreads much less easily under these circumstances. The finding that the wild-type U83 gene was present in the ES viruses, even if it was a minor component, suggested that a small amount of secreted U83 chemokine product from the frame-shifted U83 gene could cause the viruses to spread easily in the PBMCs of infants. In addition, reactivated viruses were able to spread in the PBMCs of immunosuppressed patients, even when the viruses did not encode the mature U83 gene.

It has been suggested that the variation in IE proteins might be relevant to variant divergence [15] and although

HHV-6A could use the entry receptor CD46, HHV-6B could not [29]. Although the differences of envelope glycoproteins and immediate early transactivators between HHV-6A and -6B may be mainly related to tropism of HHV-6 variants, it is possible that the difference in U83 between HHV-6 variants partly contributes to their different in vivo activities in the cell tropism of HHV-6 variants; i.e., variant A is often found in patients with central nervous system disorders [7, 30], while variant B is localized to lymphocytes [2, 3]. Interestingly, the U83 genes of variant A were conserved, even though they had no signal peptide. Therefore, the U83 gene product may function as an important intracellular signal-transduction component in HHV-6-infected cells.

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