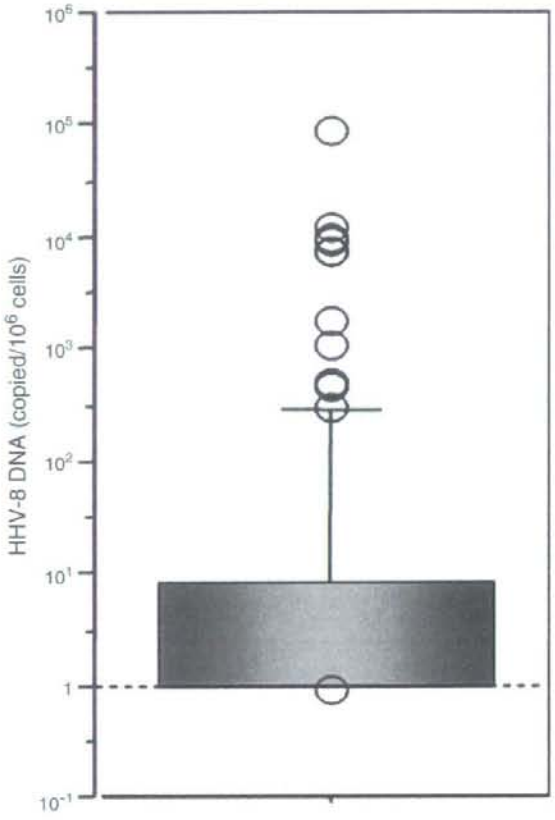

FIG. 1. Quantification of HHV-8 DNA by real-time PCR. Log_{10}-transformed HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. The dotted line shows the detection limit of the assay.

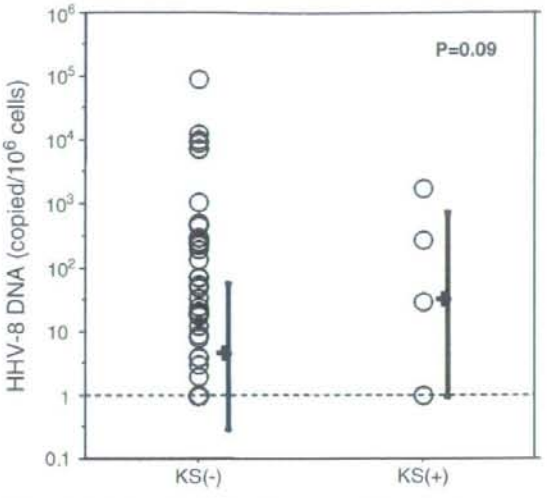

FIG. 2. HHV-8 DNA loads among the HIV-1-positive subjects with or without KS. Log_{10}-transformed HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. *p*-values were estimated by the Mann–Whitney *U* test. The dotted line shows the detection limit of the assay.

hypergammaglobulinemia was significantly higher than in subjects without hypergammaglobulinemia (Fig. 3b). HHV-8 DNA was detected in 24 of 44 (54.5%) HIV-infected subjects with hypergammaglobulinemia, whereas it was found in 13 of 81 (16.0%) subjects without hypergammaglobulinemia ($p < 0.0001$). We could not find any significant correlation between HHV-8 DNA loads and CD4-positive T cell counts.

The effect of ART on HHV-8 DNA

HHV-8 DNA load in leukocytes in subjects with ART was significantly lower than in subjects without ART (Fig. 4a). HHV-8 DNA was detected in 12 of 58 (20.7%) HIV-infected subjects with ART, whereas it was found in 25 of 67 (37.3%) subjects without ART ($p = 0.042$). There was no difference in the duration of ART between HHV-8 DNA-negative and HHV-8 DNA-positive subjects with ART (3.161 ± 2.764 vs. 3.575 ± 2.439 years, $p = 0.624$). HHV-8 DNA loads (log_{10}) per 10^6 leukocytes in subjects with a short duration of ART (<6 months) were higher than in subjects with a long duration of ART (≥6 months), but the difference was not statistically significant (0.758 ± 0.447 vs. 0.323 ± 0.111, $p = 0.309$).

Longitudinal analysis of HHV-8 DNA loads after initiation of ART

The longitudinal profiles of HHV-8 DNA loads were examined in nine subjects after initiation of ART (Fig. 4b). Within 1 month after starting ART, HHV-8 DNA transiently increased in three patients (Fig. 4b, black straight lines); in two of the three patients, KS had progressed transiently. These findings show the reactivation of HHV-8 by initiation of ART. Three months after the initiation of ART, all nine patients had achieved and maintained the decreased levels of HHV-8 DNA.

The influence of EBV on HHV-8 infection

Like HHV-8, EBV is lymphotropic and is a member of the gammaherpesvirinae; it is reactivated by HIV-1 infection. Therefore, the influence of EBV on HHV-8 infection was investigated. EBV-DNA loads (log_{10}) per 10^6 leukocytes were significantly higher in HHV-8 DNA-positive subjects (0.69 ± 0.11 vs. 1.46 ± 0.21, $p = 0.0007$) and EBV DNA was detected in 32 of 88 (36.4%) HHV-8-negative subjects, whereas they were found in 25 of 37 (67.5%) HHV-8-positive subjects ($p = 0.002$).

Relationship between HHV-8 DNA and thrombocytopenia

Figure 5 shows a case of Castleman's disease in which the platelet counts changed in inverse association with HHV-8 DNA in the leukocytes. Therefore, the association between platelet counts and HHV-8 DNA in leukocytes was examined. First, the changes in platelet counts and HHV-8 DNA were analyzed in the nine subjects before and after the initiation of ART (Fig. 6). Both the decrease in HHV-8 DNA and the

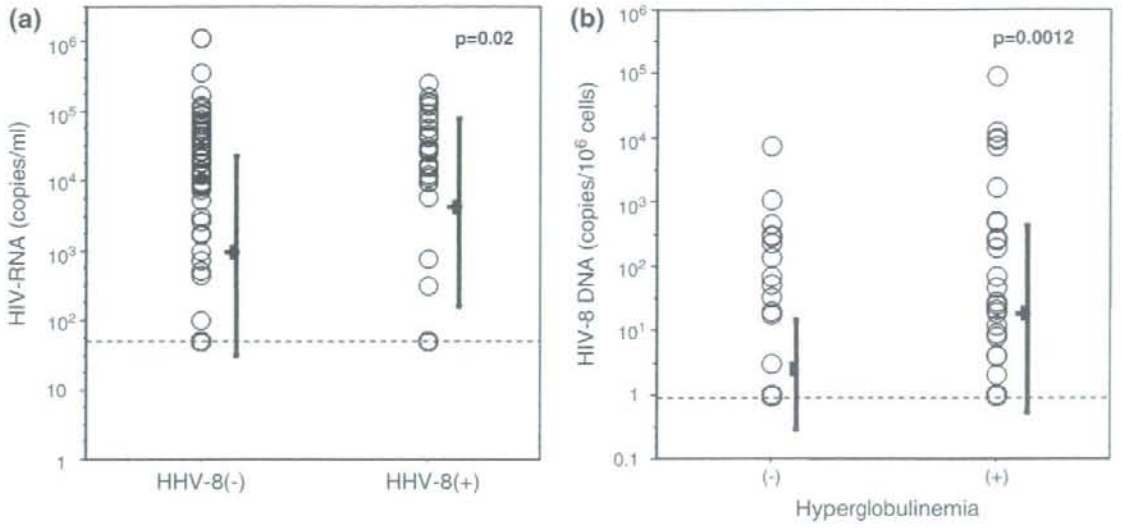

FIG. 3. The correlation between HHV-8 DNA and HIV-1-related immunovirological parameters. (**a**) HIV-RNA loads among the HIV-1-positive subjects with or without HHV-8 DNA. Log_{10}-transformed HIV-1 copy numbers/ml of serum are shown. The bar shows the mean and standard deviation. *p*-values were estimated by the Mann–Whitney *U* test. (**b**) HHV-8 DNA loads among the HIV-1-positive subjects with or without hypergammaglobulinemia. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviations. *p*-values were estimated by the Mann–Whitney *U* test. The dotted line shows the detection limit of the assay.

increase in platelet counts were significant. Furthermore, the correlation between changes in platelet counts and changes in HHV-8 DNA was obtained (Spearman; $r = -0.783$, $p = 0.0267$). Patients with HIV infection sometimes develop thrombocytopenia, and EBV is also reported to cause mild thrombocytopenia. Therefore, partial correlations were also used to assess this relationship while controlling for EBV-DNA and HIV-RNA, and the correlation between the change in HHV-8 DNA and platelet counts was obtained (partial correlation coefficient; $r = -0.733$, $p = 0.0219$).

Next, all 125 subjects with HIV infection were analyzed. There was no significant difference between the group with

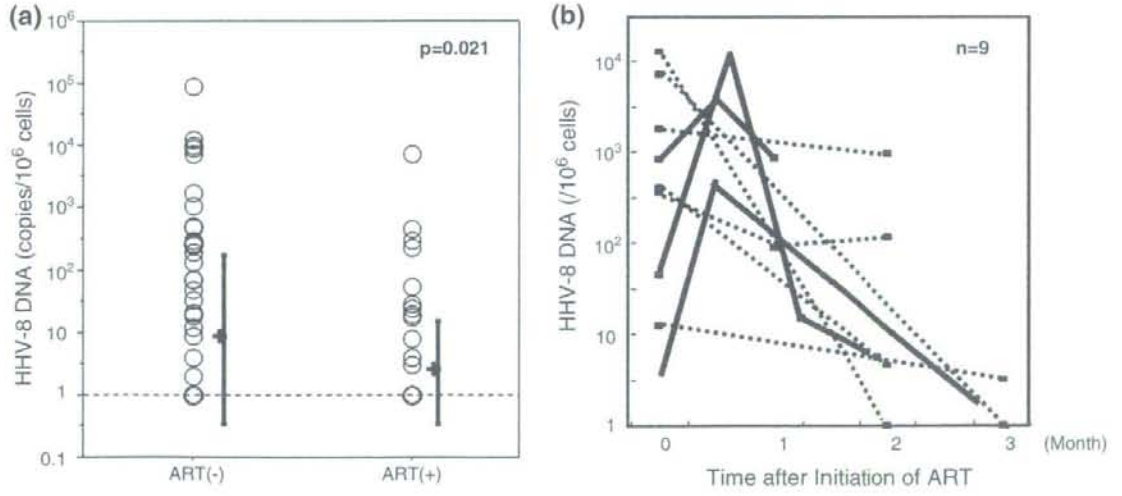

FIG. 4. The effect of ART on HHV-8 DNA. (**a**) HHV-8 DNA load in PBMCs in subjects with or without ART. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviation. *p*-values were estimated by the Mann–Whitney *U* test. (**b**) HHV-8 DNA load after initiation of ART. HHV-8 DNA loads of the nine subjects were measured at the indicated time points. The dotted line shows the detection limit of the assay.

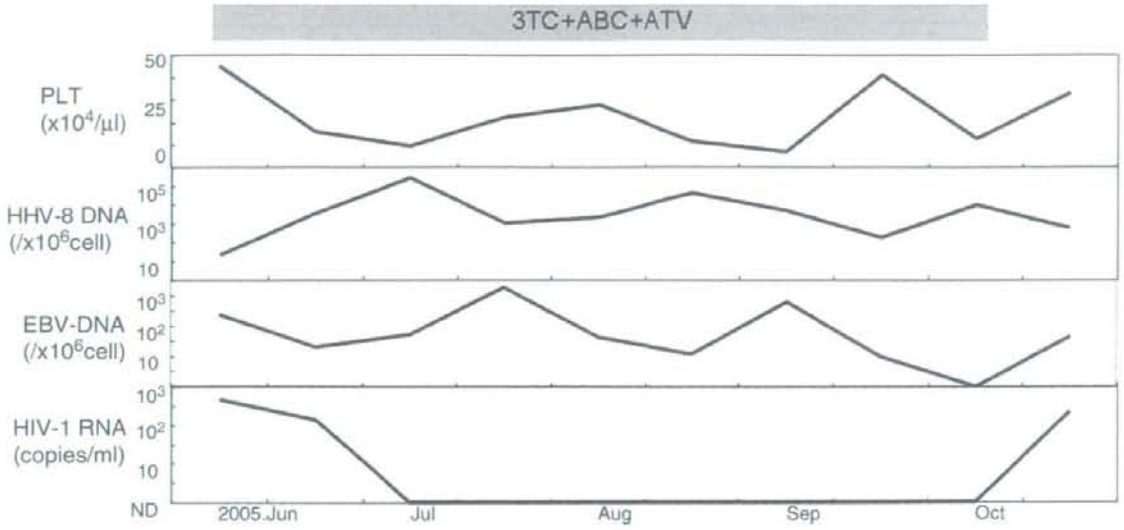

FIG. 5. Time course of the case of Castleman's disease with HIV-1 infection. Platelet counts, $\log_{10}$-transformed HHV-8 and EBV copy numbers per 10^6 leukocytes, and $\log_{10}$-transformed HIV-1 copy numbers/ml of serum are shown. The data were excerpted from Minami and Yamamoto.[10] PLT, platelet; HHV-8, human herpes virus 8; EBV, Epstein–Barr virus; 3TC, lamivudine; ABC, abacavir; ATV, atazanavir.

thrombocytopenia and the group without thrombocytopenia in terms of the number of subjects with ART, the duration of ART, and the prevalence of subjects with splenomegaly (with thrombocytopenia vs. without thrombocytopenia; 30.8% vs. 22.6%, $p = 0.520$). Similarly, no significant difference was observed between the group with HHV-8-DNA and the group without HHV-8-DNA in terms of the duration of ART and the prevalence of subjects with splenomegaly (with HHV-8 vs. without HHV-8; 26.7% vs. 22.4%, $p = 0.647$). The effects of HHV-8 DNA in leukocytes on platelet counts were examined using ANCOVA, with EBV-DNA as a covariate. ANCOVA revealed a significant interaction between EBV-DNA and HHV-8 DNA but no significant interaction between thrombocytopenia and EBV-DNA. Post hoc tests revealed that HHV-8 DNA was significantly higher in subjects with platelet counts <150,000 platelets/μl than in subjects with platelet counts ≥150,000 platelets/μl (Fig. 7a). HHV-8 DNA was detected in 11 of 14 (78.6%) subjects with low platelet counts,

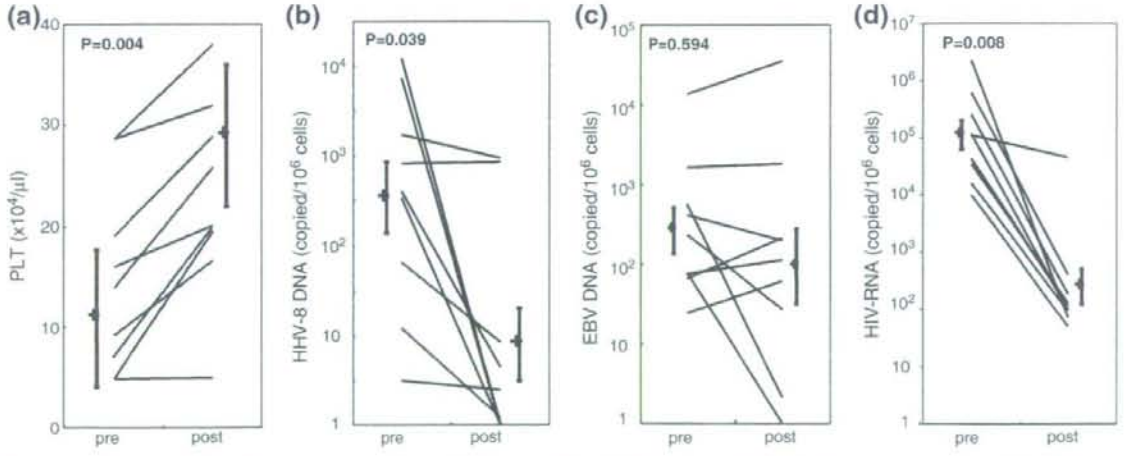

FIG. 6. Changes in the platelet counts, HHV-8 DNA loads, EBV-DNA loads, and HIV-RNA of pre- and post-ART. Platelet counts (**a**), HHV-8-DNA (**b**), EBV-DNA (**c**), and HIV-RNA (**d**) of the nine subjects were analyzed before (pre) and after 2 or 3 months of initiation of ART (post). The viral loads were $\log_{10}$ transformed. Bar shows the mean and standard deviation. p-values for the difference between the two time points were estimated by the Wilcoxon signed rank test.

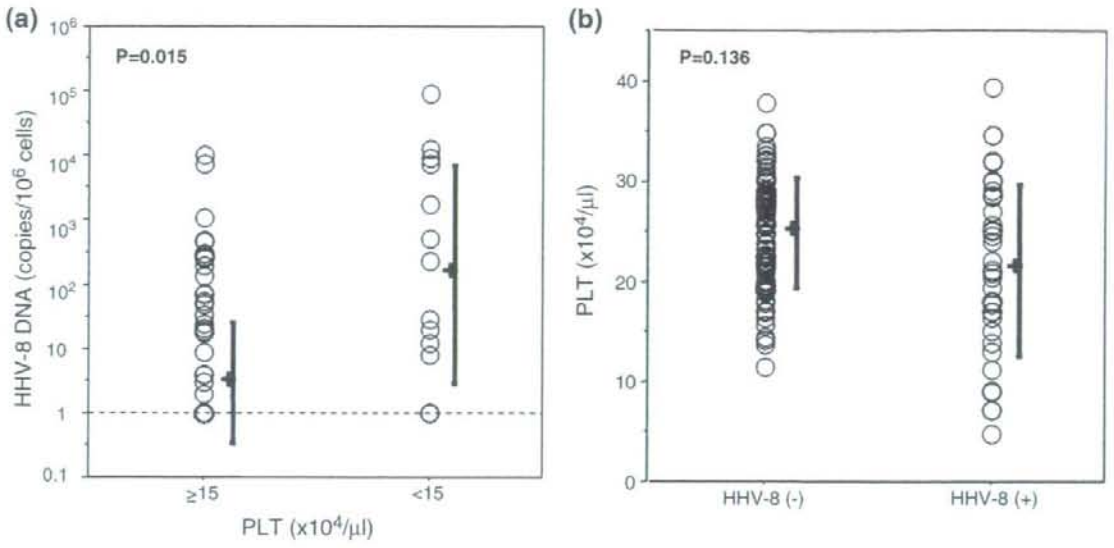

FIG. 7. Relationship between HHV-8 DNA and mild thrombocytopenia.(**a**) HHV-8 DNA load in leukocytes in subjects with platelet counts of <150,000 platelets/mm^3 or with platelet counts of ≥150,000 platelets/mm^3. HHV-8 copy numbers per 10^6 leukocytes are shown. The bar shows the mean and standard deviation. The *p*-values were estimated by a post hoc test after ANCOVA with EBV-DNA as a covariate. (**b**) Platelet counts among the HIV-1-positive subjects with or without HHV-8 DNA. The bar shows the mean and standard deviation. The *p*-values were estimated by a post hoc test after ANCOVA with EBV-DNA as a covariate. The dotted line shows the detection limit of the assay.

whereas it was found in 26 of 111 (23.4%) of subjects with normal platelet counts ($p = 0.0059$: estimated by post hoc test after ANCOVA with the prevalence of EBV-DNA as a covariate). The platelet counts were lower in HHV-8 DNA-positive subjects than in HHV-8 DNA-negative subjects, *but not significanlyt* (Fig. 7b). In subjects without ART, HIV-RNA might be a confounding factor, and thus ANCOVA was conducted with HIV-RNA and EBV-DNA as covariates.

In subjects without ART, HHV-8 DNA was also significantly higher in subjects with mild thrombocytopenia with an adjustment for EBV-DNA and HIV-RNA ($p = 0.013$, data not shown). HHV-8 DNA was not detected in the 12 subjects with autoimmune thrombocytopenia. We also examined the correlation between HHV-8 DNA loads and platelet counts in subjects without KS, because the subject with KS might have occult Castleman's disease, which can cause thrombocytopenia. HHV-8 DNA was significantly higher in subjects with platelet counts of <150,000 platelets/μl than in subjects with platelet counts of ≥150,000 platelets/μl [$\log_{10}$ (HHV-8) = 2.103 ± 1.797 vs. 0.438 ± 0.924; $p = 0.0285$]. HHV-8 DNA was detected in 9 of 12 (75.0%) subjects with low platelet counts, whereas it was found in 25 of 108 (23.1%) subjects with normal platelet counts ($p = 0.0002$). The platelet counts were significantly lower in HHV-8 DNA-positive subjects than in HHV-8 DNA-negative subjects (21.5 ± 8.61/μl vs. 25.0 ± 5.56/μl; $p = 0.1319$).

Discussion

Some previous studies have examined the prevalence of HHV-8 infection in HIV-1-infected subjects. A unique aspect of this study is that the effect of HHV-8 DNA in the peripheral blood leukocytes on HIV-1 infection was examined; a significant association between HHV-8 DNA and the clinical appearance of HIV-1 infection, especially thrombocytopenia complicated with HIV-1, was found.

Some reports have shown that the presence of KS was associated with the prevalence of HHV-8 DNA in leukocytes[14] and the severity was associated with HHV-8 DNA loads in leukocytes.[15] The current data also showed a certain association with the presence of KS and HHV-8 DNA load, but in this study there were only five subjects with KS, because the prevalence of KS in HIV-1-infected patients in Japan is low. As a result, it was impossible to determine whether the HHV-8 DNA load predicted the onset and severity of KS. To draw any conclusions concerning the clinical and prognostic utility on KS, more subjects with KS need to be analyzed.

HHV-8 encodes homologues of cytokine and cytokine response genes, such as viral interleukin-6 (vIL-6)[16] and viral interferon regulatory factor (vIRF). vIL-6 can bind the gp130 receptor to activate IL-6 response genes and promote B cell activation, which is the mechanism of hypergammaglobulinemia induced by HHV-8 infection. It was also found that HHV-8 plays an important role in hypergammaglobulinemia, which is often found in subjects with HIV-1 infection.

The current data showed that HIV-RNA load in the serum of HHV-8 DNA-positive subjects was significantly higher than that found in the serum of HHV-8 DNA-negative subjects. HHV-8 replication is activated by HIV-Tat,[17] and it has been reported that HHV-8 reactivation is associated with HIV-1 acute infection.[18] Furthermore, an open reading frame (ORF), the major transactivator of the HHV-8 lytic cycle, can also induce increased levels of HIV replication. In conclusion, these findings show that HHV-8 coinfection is associated with

immunological characteristics and disease severity of HIV infection.

ART reduced HHV-8 DNA loads as well as HIV-RNA (Fig. 4). The inhibition of HIV-1 replication with ART probably leads to the reconstitution of the immune system and regenerates effective immune responses against HHV-8. Moreover, protease inhibitors block the production of inflammatory cytokines,[19] which in turn may result in the downregulation of HHV-8 replication. In this longitudinal study during ART, HHV-8 DNA loads transiently increased in three subjects soon after the initiation of ART. This might be due to the disorder of cytokine balance, which occurs transiently in the process of the reconstitution of the immune system, and this might be one of the explanations for the immune reconstitution syndrome of KS. Some inflammatory cytokines are induced in immune reconstitution syndromes. It has recently been reported that some cytokines, which are associated with the development of KS, were elevated in other reconstitution syndromes.[20] It is possible that reactivation of HHV-8 by ART might activate the inflammation of the other reconstitution syndromes by the increase in vIL-6 or other cytokines.

The difference between HHV-8 DNA in the subjects with platelet counts of $<150{,}000$ platelets/μl and in the subjects with platelet counts of $\geq 150{,}000$ platelets/μl was significant, but the difference between the platelet counts in the HHV-8 DNA-positive subjects and the platelet counts in the HHV-8 DNA-negative subjects was not significant. This is because HHV-8 DNA correlates with "mild" thrombocytopenia.

This is the first report that analyzed the association between HHV-8 DNA and thrombocytopenia in HIV-1-infected subjects, though there are a few studies that showed the relationship between HHV-8 infection and hematological disorders[21] or posttransplantation bone marrow failure in HIV-1-negative subjects.[22] Some case reports have described thrombocytopenia complicated with HHV-8-induced Castleman's disease, hemophagocytic syndrome, or other inflammatory syndromes. The HIV-1-positive subjects sometimes develop thrombocytopenia, which has been thought to be caused by an autoimmune mechanism. Recently, antibody cross-reactivity between a known epitope region of HIV-1 proteins and platelet GPIIIa49-66 has been reported to be responsible for HIV-1-associated thrombocytopenia.[23] However, the present study showed that HHV-8 also contributes independently to the thrombocytopenia complicated in HIV-1 infection.

The molecular mechanism of thrombocytopenia induced by HHV-8 remains to be established, but it is possible that some cytokines or chemokines induced by HHV-8 might be involved in thrombocytopenia. For example, interleukin-8 (IL-8), which is induced by HHV-8 infection,[24] binds the receptors on megakaryocytes and platelets and inhibits megakaryocytopoiesis.[25] Furthermore, HHV-8 is reported to infect CD34-positive hematopoietic progenitor cells[26,27]; HHV-8 harboring CD34-positive cells might also contribute to hematological disorders including thrombocytopenia.[28] Another possible explanation for thrombocytopenia is an autoimmune mechanism such as immune thrombocytopenia (ITP). Chronic stimulation of the B cell clones induced by HHV-8 could favor the production of autoantibodies, some of which might react against platelet. In fact, Evan's syndrome complicating multicentric Castleman's disease[29] and ITP complicating KS have been reported.[30]

In Italy, the area of subendemicity of HHV-8, the presence of HHV-8 DNA in HIV-1-negative elderly people has been reported to be associated with mild thrombocytopenia.[31] In Japan, HHV-8 infection in HIV-1-negative subjects is rare; as a result, when the 12 subjects with autoimmune thrombocytopenia were examined, the association between HHV-8 and autoimmune thrombocytopenia in HIV-1-negative subjects was not distinct. However, the fact that HHV-8 DNA was not detected in the HIV-1-negative subjects with autoimmune thrombocytopenia suggests that HHV-8 is not a main causative agent of autoimmune thrombocytopenia.

In conclusion, a significant association was observed between HHV-8 infection and the clinical appearance of HIV-1 infection, especially in regard to mild thrombocytopenia, hypergammaglobulinemia, and disease severity. It is possible that some of these effects of HHV-8 infection are related to other independent etiological cofactor or some other viruses. However, it may be useful to monitor HHV-8 DNA in leukocytes of HIV-1-positive subjects to assess and predict disease severity and to select the optimal treatment modalities.

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Disclosure Statement

No competing financial interests exist.

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原　著

HIV 感染者における歯科医療連携に関する研究

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目的：HIV 感染者の歯科診療における連携に関して仙台，東京，名古屋，大阪，福岡のブロック拠点病院地区の一般歯科医院にアンケート調査を行い，患者の受け入れを阻害する要因について検討する。

対象・方法：HIV 感染者の歯科治療，院内感染対策について共通のアンケート用紙作成。各地域の一般歯科医院に郵送し，得られた回答 1,462 件を分析した。

結果：HIV 感染者が受診した時の対応について，332 名（23.8%）の歯科医師が行うと回答した。しかし，1,011 名（72.5%）は拠点病院を紹介すると回答し，断るとの回答も 51 名（3.6%）に認められた。治療困難な理由としては，院内感染対策が不十分であるとの回答が圧倒的に多く，その他歯科治療に対する情報不足，スタッフが嫌がるなどが上げられた。院内感染対策について，48.9% の歯科医院ではマニュアルが作成されていなかった。ハンドピースの滅菌については，全ての患者に行うと回答した歯科医院は 256（18.6%）であった。診療時の手袋の装着について必ず装着するとの答えは，795（57.0%）であり，年齢の上昇とともに装着率も低下した。

結論：今回の結果から，院内感染対策の不備，歯科治療に対する情報不足が，HIV 感染者の歯科治療を困難としており，今後，スタンダードプリコーションの徹底，HIV 歯科医療に対する情報の公開，拠点病院を核とした研修，さらには行政側からの経済的支援が連携を進めていく上で必要であると思われた。

キーワード：HIV 感染者，歯科治療，歯科医療連携，院内感染対策

日本エイズ学会誌 **10**：41-49，2008

1. 研究目的

HIV 感染者の歯科医療体制は，拠点病院を中心に整備されてきた。しかし，すべての拠点病院に歯科が存在するわけでなく，一部の歯科に患者が集中する傾向が強い。HIV 感染者が身近で歯科医療を受けられるようにするには，各地域で一般歯科医院との連携による歯科医療体制が整備されることが必要である。しかし，HIV 感染者が増加しているにもかかわらず，受け入れが少ないのが現状であり連携は進んでいない。

今回，歯科医療連携に関してブロック拠点病院が存在する仙台，東京，名古屋，大阪，福岡の各地区の患者受け入れ体制について，歯科医療連携を阻害する要因を検討し，その問題点を明確にするため，一般歯科医院を対象にアンケート調査を行った。

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2. 研究方法

開業形態，HIV 感染症，感染者の歯科治療，院内感染対策について共通のアンケート用紙を作成した（表 1）。平成 15 年 11 月から平成 16 年 1 月に各ブロック拠点病院歯科口腔外科を通じて各地域の一般歯科医院に郵送し返却された回答を集計，分析した。なお，今回の研究は厚生労働省国立病院エイズ医療共同研究費を用いて行ったため，調査対象は国立ブロック拠点病院とした。さらに，アンケート調査を行った一般歯科医院は，各ブロック拠点病院が存在する地域の歯科医師会に所属する歯科医院を対象とした。

3. 結　果

各地域のアンケート調査を郵送した一般歯科医院数とアンケート回収数は，仙台地区（宮城県）：アンケート郵送数 993 歯科医院，回収数 259 件（回収率 26.0%）。東京地区（新宿，杉並区）：アンケート郵送数 755 歯科医院，回収数 309 件（回収率 40.9%）。名古屋地区（名古屋市）：アンケート郵送数 506 歯科医院，回収数 218 件（回収率 43.1%）。大

表 1 HIV 感染者の歯科治療に関するアンケート

1. 先生の年齢　　歳　　性別　男性　　女性
2. 開業期間　1) 5年未満　2) 5～10年　3) 10～15年　4) 15～20年　5) 20年以上
3. 歯科医師数（常勤医）（　　）人
4. 歯科衛生士数（　　）人
5. ユニット台数（　　）台
6. B型，C型肝炎患者の歯科治療をおこなっておられますか
   1) している　2) していない　3) わからない
7. 感染症（HB, HCV など）を有する患者の治療の際，ユニット，治療の時間帯を特別に決めていますか
   1) 決めている　2) 決めていない　3) わからない

Ⅰ HIV 感染症について，お聞きします。

1. HIV 感染症について
   1) よく知っている　2) 知っている　3) 余り知らない　4) 知らない
2. 日本での HIV 感染者数について
   1) 増加している　2) 変わらない　3) 減少している　4) 分からない
3. 感染経路について
   1) 知っている　2) 知らない
4. 唾液も主な感染経路の1つと考えますか
   1) そう思う　2) 思わない　3) 分からない
5. HIV の感染力は B 型肝炎より弱いことをご存じですか
   1) 知っている　2) 知らない
6. HIV に関連した研修会，講演会に出席したことがある
   1) ある　2) ない

Ⅱ HIV 感染者の歯科治療について，お聞きします。

1. HIV 感染者の歯科治療の経験がある
   1) ある　2) ない
2. HIV 感染者が貴医院を受診した時，どのように対応されますか
   1) 治療する　2) 断る　3) 地区の拠点病院を紹介する
3. 貴医院にて治療が困難である場合その理由はなんですか，下記の項目から2つ選んでください。
   1) HIV 感染についてよく知らない
   2) HIV 感染者の歯科治療についての情報がない
   3) HIV 感染者の治療に対する院内感染対策が不十分である
   4) スタッフがいやがる
   5) 怖い
   6) 他の患者で忙しく，時間が取れない
   7) その他（　　）
4. 将来歯科治療を必要とする HIV 感染者が増加した場合，貴医院で治療を行いますか
   1) 行う　2) できれば行う　3) 行わない　4) わからない
   ＊行うためには，どのようなことが必要と考えますか

Ⅲ 院内感染対策について，お聞きします。

1. 貴医院で消毒，滅菌についてマニュアルを作成しておられますか
   1） している　2） 現在作成中　3） していない
   4） その他（　　）
2. ハンドピースの滅菌をされますか
   1） 全ての患者に行う　2） 2回/日程度　3） 1回/日程度
   4） 感染症患者の時だけ　5） しない
   6） その他
3. エアータービンに逆流防止装置を設置していますか
   1） している　2） 一部している　3） していない
4. 印象物の消毒はされていますか
   1） 全てしている　2） 感染症患者だけ　3） していない
   4） その他（　　）
5. 診療時手袋はされますか
   1） 必ずする　2） 感染症の患者の時のみする　3） しない

Ⅳ その他，御意見がありましたらお願いします。

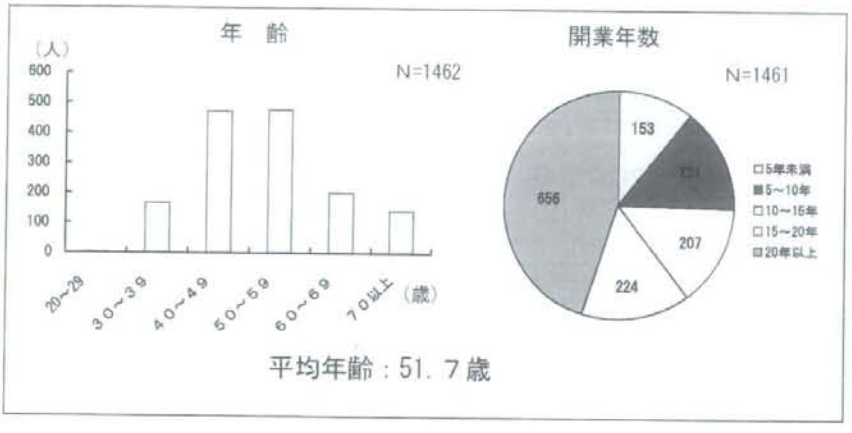

図 1　年齢と開業年数

阪地区（大阪市）：アンケート郵送数 530 歯科医院，回収数 210 件（回収率 39.6%）。福岡地区（福岡市）：アンケート郵送数 947 歯科医院，回収数 466 件（回収率 49.2%）であった。総アンケート郵送数は 3,731 歯科医院，アンケート回収数は 1,462 件（回収率 39.2%）である。

① 年齢と開業形態

対象の年齢層は 50 歳代が 478 名（32.7%），40 歳代が 472 名（32.3%）であり 40～50 歳代で全体の 65% を占めており平均年齢は 51.7 歳であった。開業年数は 20 年以上が 656 名，15 年から 20 年までが 224 名であり，15 年以上で全体の 60.2% を占めていた（図 1）。診療所の規模は歯科医師数 1 人が 70.8%（1,019 歯科医院），2 人が 346 歯科医院（24.0%）で 94.8% は 2 人以下であった。また，1 歯科医院の診療台（ユニット）数は平均 3.30 台で 67.0% は 3 台以下

であった。感染症を有する患者の治療の際，ユニット，治療の時間帯を決めていますかの設問に対して，決めているは 461 名（32.5%），決めていないは 870 名（61.3%）であった（図 2）。

② HIV 感染症について

HIV 感染症については，1,257 名（87.1%）の歯科医師から，よく知っている，知っているとの回答が得られた。日本での感染者数についても 1,352 名（93.4%）の歯科医師は増加しているの知識を持っていた。感染経路については，知っているが 1,366 名であり，知らないは 70 名であった（図 3）。唾液も主な感染経路の 1 つと考えますかとの設問に対しては，そう思うは 492 名（34.3%），思わないが 775 名（54.0%），分からないが 167 名（11.6%）であった。また，感染力に関して 1,221 名（84.7%）の歯科医師は HIV は HBV より感染力が弱いことを知っていた。HIV に関する情報源として，HIV に関連した研修会，講演会への出席の有無について尋ねたところ，出席したことがある歯科医師は 649 名（45.1%）であった（図 4）。

③ HIV 感染者の歯科治療について

HIV 感染者の歯科治療経験がある歯科医師は，1,462 名中 71 名（仙台：11 名，東京：23 名，名古屋：6 名，大阪：12 名，福岡：19 名）で全体の 4.8% であった。HIV 感染者が受診した時の対応については，治療するは 332 名（23.8%）であったが，1,011 名（72.5%）は拠点病院を紹介すると回答し，断るとの回答も 51 名（3.6%）に認められた（図 5）。HIV 感染者の歯科治療が困難である理由としては，回答を得られた 1,914 項目中，院内感染対策の不十分が 754 回答と最も多く，次に HIV 感染症の歯科治療についての情報がない 409，スタッフがいやがるが 333，他の患者で忙しく時間が取れない 158，怖い 105 の回答であった。その他と

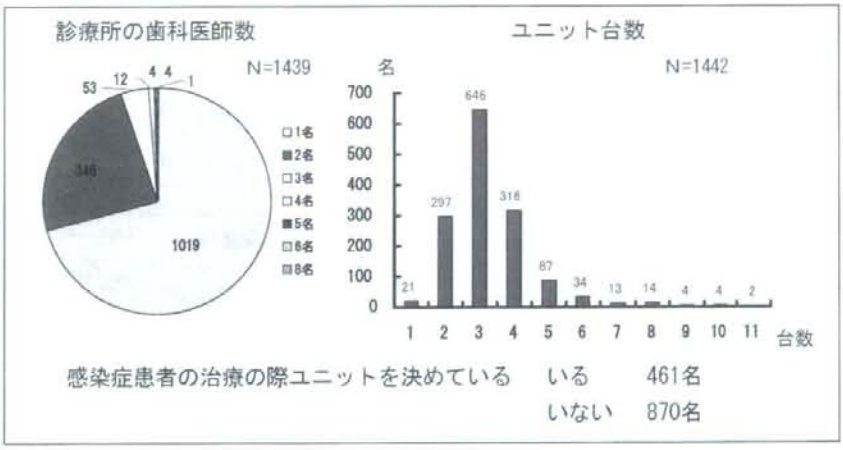

図 2 開業形態について

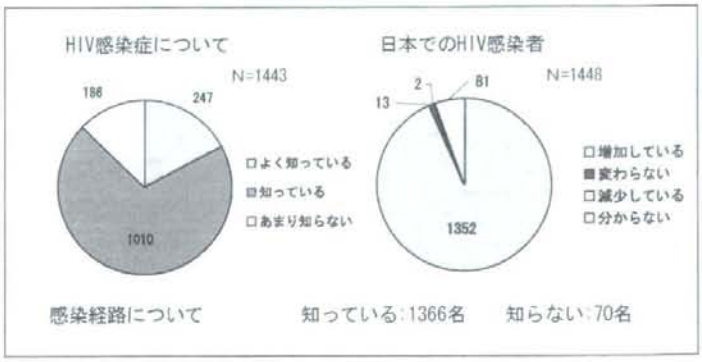

図 3 HIV 感染症に関する知識 1

しては風評被害がでる，治療にストレスを感じるなどの回答があった（図6）。将来歯科治療を必要とするHIV感染者が増加した場合，貴医院で治療を行いますかの質問に対して，557名（39.1%）の歯科医師は行う，できれば行うの意思表示が見られた。しかし，行わないの回答も420名29.5%認められた（図5）。治療を行うために必要なことと

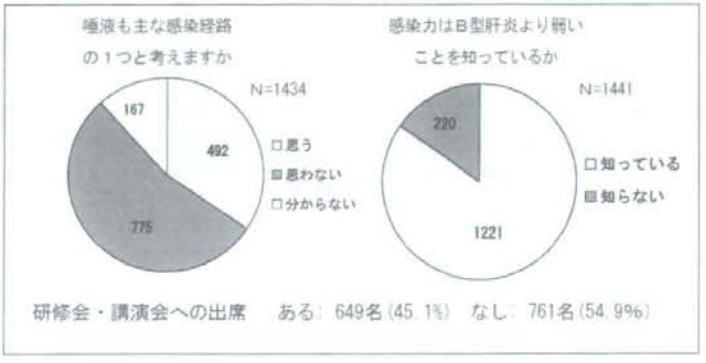

図 4　HIV感染症に関する知識2

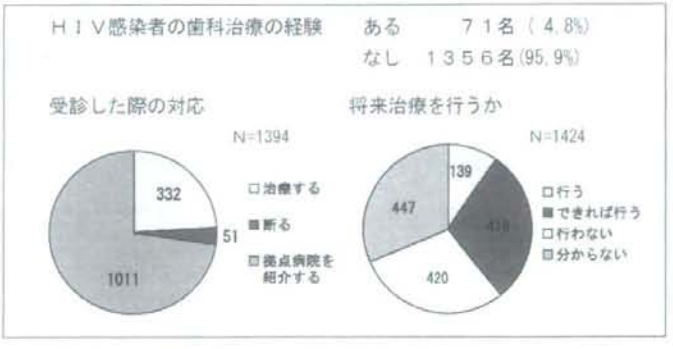

図 5　HIV感染者の歯科治療について

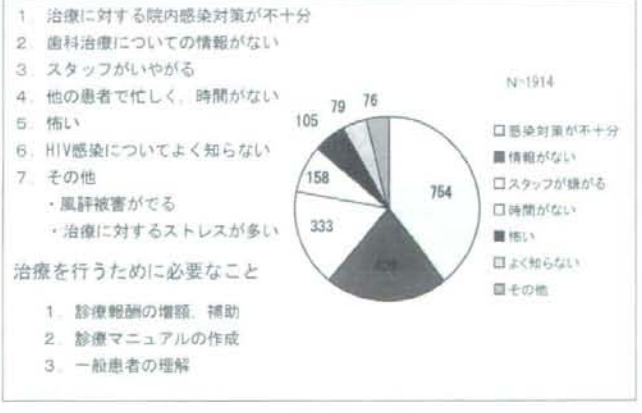

図 6　治療が困難な理由

しては，院内感染対策実施のため診療報酬の増額が必要との回答が最も多く，診療マニュアルの作成が必要との回答も多くあった。

④ 院内感染対策について

まず，開業形態の設問でB，C型肝炎患者の歯科治療を行っていますかの設問に対して，行っていない126名に対し1,160名の歯科医師から行っているの回答が得られた。消毒・滅菌マニュアルを作成しているところは，あるが589歯科医院（41.6%）であり，なしが693（48.9%），作成中が97歯科医院であった。ハンドピースの滅菌については，全ての患者に行うと回答した歯科医院は，256（18.6%）であり，1日1回が245（17.8%），1日2回が127（9.3%）であった。518（37.8%）歯科医院は感染症患者の時だけ行っており，行わないとの回答も225（16.4%）歯科医院から得られた。血液・唾液の逆流を防止するタービンの逆流防止装置を設置している施設は，全体の599（42.1%）であり607（42.7%）歯科医院は設置していなかった（図7）。診療時の手袋の装着については，必ず装着するとの答えは，回答があった1,394歯科医院中795（57.0%）であった。感染者の時のみ着用が478（34.3%），着用しないとの回答も136（9.7%）認められた。年齢と手袋の装着の関係について調べてみると，40代，50代，60代と年齢が増すにつれて装着率も低下しており，50歳代では通常装着しない割合が50%を越え，60代では75%が通常手袋を装着せず診療を行っていることが判明した（図8）。

5. 考　　察

日本全国にエイズ診療拠点病院は366病院あるが，そのうち歯科診療において外来患者を含めて治療可能な病院は209病院のみであり，地域に複数施設ないところがほとん

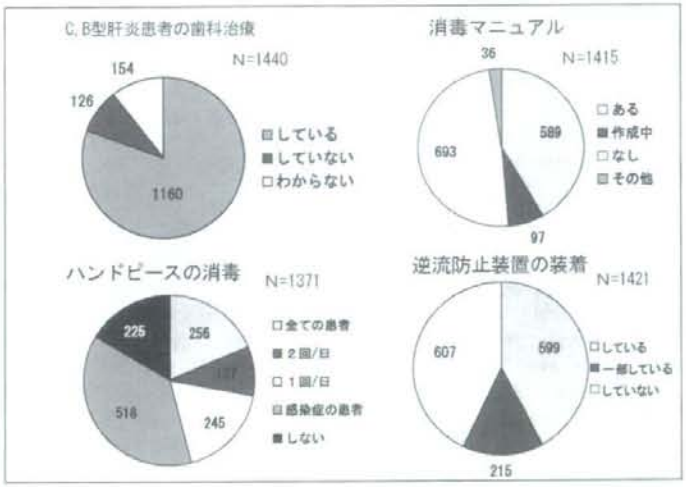

図7　院内感染対策

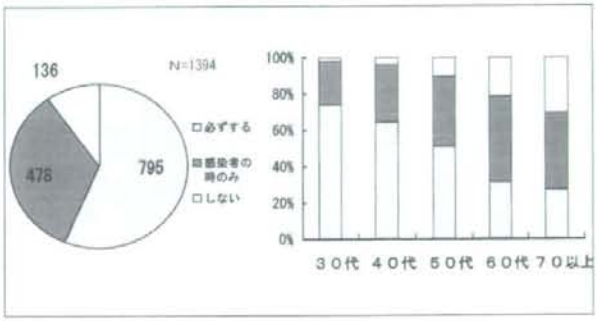

図8　診療時の手袋の装着

どである。したがって，HIV 感染患者に対する歯科医療の対応の確立は急務であり，HIV 感染者が身近で歯科医療を受けられるようにするには，各地域で一般歯科医院との連携による歯科医療体制が整備されることが必要である。

今回のアンケート調査は対象が大都市圏の一般歯科医院である。アンケート項目の分析で地域の特殊性による調査結果の大きな相違は認められなかったため，全体として分析した。対象歯科医師の平均年齢は 51.7 歳であり，50 歳代と 40 歳代にピークを持つ分布となっていた。この点に関しては，厚生労働省平成 16 年医師・歯科医師・薬剤師調査の報告[1]によると，一般診療所歯科医師の平均年齢 49.1 歳であり，年齢階級が 40 歳代，50 歳代が多い分布と報告しており，平均年齢が若干上回っているが同じ傾向が認められた。開業形態としては，歯科医師数は 70% が 1 人であり，平均 3.3 台の診療台を使用し診療している。感染症を有する患者の治療の際，61% の歯科医師はユニットを特に決めず治療を行っている。このことは，スタンダードプリコーションが実践されていれば問題ないが，今回のアンケート調査にて院内感染対策の不十分を指摘する回答が多く，さらにハンドピースの滅菌を全ての患者に行っている割合が 18.6% のみの状態においては感染拡大の要因にもなり問題があると思われる。しかし，多くの歯科医院は歯科医師 1 人，ユニット 3 台で診療している診療背景を考えると，患者が込んでいる時に感染症の患者の治療のためにわざわざユニットを別に空ける余裕がないことが推測される。そのため歯科のような小規模の開業歯科医院こそスタンダードプリコーションの概念を取り入れ積極的に感染予防を行う必要があると考える。

HIV 感染に関する一般的な知識は，今回アンケート調査を行った大都市圏一般歯科医院の約 90% の歯科医師は持っていることが確認された。ただし，医療従事者に必要な HIV 感染に関する具体的な知識を得るために必要な HIV に関連した研修会，講演会への参加経験者は，649 名（45.1%）であった。HIV の感染力に関して 84.7% の歯科医師は HBV より感染力が弱いことを知っていても，現在，血液を含まない唾液は感染源とは考えられていないが，492 名（34.3%）は唾液を主な感染経路の 1 つと考えていた。一般的な知識はあっても，実際の診療を行う上で必要な知識は十分でないことが推測される。講演会，研修会への参加が少ないことは，研修会，講演会の機会が少ないためか，機会はあるが患者が受診するかもしれないという危機感がまだ十分に認識されておらず関心度が低いためか今回の調査では明らかでない。しかし，今後は，行政，歯科医師会が中心となり定期的な講演会，研修会の開催，参加の推進が必要であると思われる。

HIV 感染者の歯科治療経験がある歯科医師は 71 名（4.8%）であった。五島[2]らは平成 16 年の歯科医師 435 人からのアンケート調査で 6% に経験があったと報告している。しかし，今回の調査で日本の感染者の 7 割が居住している関東甲信越地域の中でさらに感染者が多く居住する新宿区を含む東京地区の調査でも 23 名 7.4% である。感染を申告せず受診している可能性もあり，森崎[3]，五島ら[4]も感染の黙秘を指摘しており，実際の経験はもっと高いかもしれない。東京に比べ感染者数が少ない福岡で 19 名経験のある歯科医師がいることが判明した。今後の連携を進める上で，どのような経緯で診療したのか非常に興味があるが調査では明らかにすることはできなかった。HIV 感染者が受診した時，332 名（23.8%）の歯科医師が治療すると回答した。また，将来感染者が増加した場合，診療するとの回答も 557 名（39.1%）あったことは，今後の歯科連携を進める上で希望がある数字と考える。しかし，多くは断る，拠点病院を紹介するとの回答であり 1,062 名 76.1% を占めている。治療困難な理由としては，院内感染対策が不十分であるとの回答が 754 と圧倒的に多かった。歯科治療に対する情報不足が 409，スタッフが嫌がる 333 との回答も多い。また，怖いとの回答も 105 見られた。相沢ら[5]の調査でもほとんどの歯科医師は実際に治療することを躊躇しており，その原因として安全でない，適切な感染予防の訓練を受けていないことを挙げている。前述したように HIV に関する漠然とした知識は持っているものの，実際の治療に必要な知識が不足している。中野ら[6]国立国際医療センターでの歯科医療従事者への HIV に関する研修を通じて，研修後には「HIV 感染症は特別でない」など認識の変化を報告しており，歯科医師だけでなく歯科衛生士を含めたスタッフへの教育，HIV に関する正確な情報の伝達が重要であると思われる。

一般に HBV 対策ができていれば HIV 感染者の歯科治療は可能であると理解される。1,160 名（80.5%）は B，C 型肝炎患者の歯科治療を行っている。しかし，アンケート結果では，84.7% の歯科医師は HIV の感染力は HBV より弱いことを知っているにもかかわらず，感染力の弱い HIV 感染者に対し感染対策が不十分であるとの回答であった。どのような感染対策を行っているのであろうか。一般歯科医院の 48.9% は消毒マニュアルを完備していない。マニュアルが存在しない状態では，ハンドピースの滅菌，印象物の消毒に関してその内容にばらつきがある。歯科治療において患者の血液に汚染されるハンドピースの滅菌に関しては，全ての患者に行うとの回答は 256 名（18.6%）であり，感染症患者の時のみ滅菌するとの回答は 518 名全体の 37.8% を占めている。患者が感染症を持っていると申告するとは限らない。行わない 222 名（16.4%）を加えると実に今回アンケートを行った一般歯科医院の 54.2% は通常ハンド

ピースの滅菌を行っていないことは驚きである。手袋の装着に関しても，未だに装着しないが136名（9.7%），感染者のみ装着が478名（34.3%）であり，通常の診療では44%の歯科医師は手袋を装着せず診療していることが明らかとなった。50歳代では通常装着しない割合が50%を越え，60代では75%が通常手袋を装着せず診療を行っている。50歳以降の年齢では大学での教育で手袋装着をした歯科治療の教育を受けておらず，また根管治療など細かい治療に関しては手先の感覚で手袋を装着するとやりにくいこともあり装着率が低いことが推測される。今後，一般歯科を支えている40代以降の歯科医師への院内感染対策の教育がHIV感染者の治療を行う以前の歯科全体の問題として重要であると思われる。また，大学での卒前教育も重要であり，山中ら[7]は学生教育でHIV感染を含めた感染症，感染対策の講義枠を増やすことを提唱している。スタンダードプリコーションの徹底のためには，マニュアルの作成がどうしても必要である。平成19年4月より「改正医療法」が施行され，無床診療所においても医療安全管理体制の充実・強化が要求されている。院内感染予防対策はその中の大きな柱の1つであり，マニュアル作成は必須である。アンケート調査を行った時期以降，歯科医師，歯科衛生士向けにHIVに関連した感染予防の本[8-10]も出版されており，マニュアルの作成は容易になり完備されると思われる。今後はその内容の実施に向け，日本歯科医師会，各都道府県の行政を中心とした研修会，講習会の開催が望まれる。日本HIV歯科医療研究会では平成19年から各HIV拠点病院歯科の院内感染対策における研修事業を開始しており，ブロック拠点病院，拠点病院を通じた一般歯科医院への研修会も今後は可能になると思われ，さらに院内感染予防対策の実施が進むと予想される。

ハンドピースの滅菌，エアータービンの逆流防止装置の装着は予算面からその更新，数を揃えることが十分に行われておらず，設備に対する財政面での援助が必要であると思われた。記述回答から各地区とも経済的負担から歯科診療報酬に対する不満が非常にたくさん寄せられた。山口ら[11]の報告では1回のHIV感染者歯科治療に使用するガウン，フェイスシールドマスク，手袋等を合わせて最低合計750円かかる現実を考えると，歯科医師の社会的責任だけでは進んでやりますとは言えない現状があり，障害者加算のような診療報酬面での工夫も必要であると考えられた。

HAART療法が導入されHIV感染症の予後は大幅に改善され致死的疾患から慢性疾患の仲間入りをした。十分に管理された状態では，血液中ウイルス量も検出以下または少なく，感染対策も以前にくらべ容易になったと思われる。医療連携を進める上で別の重要なことは，患者のプライバシーが守られるかである。患者は，自分の病状が他人に漏れないか大きな不安を持っており，医療従事者は患者のプライバシーの保護に十分配慮する必要がある。治療の際患者のプライバシーが守られ，適切な患者医療情報と，歯科医師の正しい知識があれば治療は一般歯科医院で十分対応が可能である。今回のアンケート回収率は39.2%であり，未回答の60.8%はHIV歯科医療に興味のない歯科医師層が多く含まれ，実際のHIV感染症に対する知識，歯科治療への対応はアンケート結果より低いことが推測される。今後の患者の増加を考えると医療連携は必須であり，興味のない歯科医師層も含めた講習会，研修会参加の推進，正しい医療情報の伝達，診療報酬の改善が今後の連携による歯科医療体制の整備を進める上で特に重要と思われた。今回のアンケート調査以降歯科における医療環境は大きく変化しており，この分析結果を基に今後再度アンケート調査を実施し，結果を比較分析することで新たな問題点が明らかにできれば，その解決のための方法を検討しさらに連携を進める上で有用であると思われる。

今後，各地域でHIV感染者の歯科治療を一般歯科医院との連携をより進めるためには，①スタンダードプリコーションの確立のため，各歯科医師会が共通の消毒マニュアルを作成する。②定期的な院内感染対策の研修会の実施，参加の推進。③歯科医師会会報，歯科医療雑誌，医療新聞などを通じたHIV歯科医療に対する情報の公開。④歯科診療報酬での院内感染対策予防費の加算の設定。⑤拠点病院に対する人的補充，財政的援助。これらが必要であると考えられた。

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A Study of the Dental-care Collaboration System for HIV Infected Patients

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Objective : The purpose of this study was to conduct a questionnaire survey on dental care cooperation for HIV patients at general dental clinics in major hospital block areas, including Sendai, Tokyo, Nagoya, Osaka, and Fukuoka, and to investigate the factors inhibiting the acceptance of such patients.

Subjects and Methods : A questionnaire survey regarding knowledge of HIV, dental care of patients, and infection control was conducted, and the answers obtained—including 1,462 cases at general dental clinics—were analyzed.

Results : Regarding how to respond to the situation when HIV patients visit, 332 (23.8%) general dentists answered that they would see the patients. But, 1,011 (72.5%) general dentists answered that they would ask for the dental treatment of the patents from major hospital block areas. 51 (3.6%) general dentists answered that the treatment would be refused. As for reasons why care is difficult, the answer from the vast majority at general dental clinics was that infection control is not sufficiently covered, and other answers included a lack of information about dental care, thus also indicating staff were unwilling. As for infection control, no manuals had been created in 48.9% of all surveyed dental clinics. With regard to sterilizing handpieces, 256 (18.6%) general dental clinics answered that sterilization is carried out for all patients. As for wearing gloves in practice, 795 (57.0%) general dentists responded that they always wear gloves. The wearing of gloves decreased with age.

Conclusion : From these results, it appears that inadequate infection control and a lack of information about dental care make dental care for HIV patients difficult, and comprehensive standard precautions, the disclosure of information for HIV dental care, training focused on major hospitals, and furthermore, increased financial support from the government are required in order to promote cooperation in this matter.

Key words : HIV patient, dental care, cooperation of dental care, infection control

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Non-Cleavage Site Gag Mutations in Amprenavir-Resistant Human Immunodeficiency Virus Type 1 (HIV-1) Predispose HIV-1 to Rapid Acquisition of Amprenavir Resistance but Delay Development of Resistance to Other Protease Inhibitors▽

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In an attempt to determine whether mutations in Gag in human immunodeficiency virus type 1 (HIV-1) variants selected with a protease inhibitor (PI) affect the development of resistance to the same or a different PI(s), we generated multiple infectious HIV-1 clones carrying mutated Gag and/or mutated protease proteins that were identified in amprenavir (APV)-selected HIV-1 variants and examined their virological characteristics. In an HIV-1 preparation selected with APV (33 passages, yielding HIV_{APVp33}), we identified six mutations in protease and six apparently critical mutations at cleavage and non-cleavage sites in Gag. An infectious recombinant clone carrying the six protease mutations but no Gag mutations failed to replicate, indicating that the Gag mutations were required for the replication of HIV_{APVp33}. An infectious recombinant clone that carried wild-type protease and a set of five Gag mutations ($rHIV_{WTpro}^{12/75/219/390/409gag}$) replicated comparably to wild-type HIV-1; however, when exposed to APV, $rHIV_{WTpro}^{12/75/219/390/409gag}$ rapidly acquired APV resistance. In contrast, the five Gag mutations significantly delayed the acquisition of HIV-1 resistance to ritonavir and nelfinavir (NFV). Recombinant HIV-1 clones containing NFV resistance-associated mutations, such as D30N and N88S, had increased susceptibilities to APV, suggesting that antiretroviral regimens including both APV and NFV may bring about favorable antiviral efficacy. The present data suggest that the preexistence of certain Gag mutations related to PI resistance can accelerate the emergence of resistance to the PI and delay the acquisition of HIV resistance to other PIs, and these findings should have clinical relevance in the therapy of HIV-1 infection with PI-including regimens.

Combination antiretroviral therapy using reverse transcriptase inhibitors and protease inhibitors (PIs) produces substantial suppression of viral replication in human immunodeficiency virus type 1 (HIV-1)-infected patients (3, 27, 28, 42). However, the emergence of drug-resistant HIV-1 variants in such patients has limited the efficacy of combination chemotherapy. HIV-1 variants resistant to all of the currently available antiretroviral therapeutics have emerged both in vitro and in vivo (6, 16, 27, 30). Of note, a number of PI resistance-associated amino acid substitutions in the active site of protease have been identified, and such substitutions have considerable impact on the catalytic activity of protease. This impact is reflected by impaired processing of Gag precursors in mutated-protease-carrying virions and by decreased catalytic efficiency of the protease toward peptides with natural cleavage sites (7, 29, 31, 43).

However, the highly PI-resistant viruses frequently have amino acid substitutions at the p7-p1 and p1-p6 cleavage sites in Gag. These mutations have been identified in PI-resistant HIV-1 variants selected in vitro (2, 5, 8, 29) and in HIV-1 isolated from patients with AIDS for whom chemotherapy including PIs was failing (26, 40, 47, 48). These mutations are known to compensate for the enzymatic impairment of protease, per se, resulting from the acquisition of PI resistance-conferring mutations within the protease-encoding region. Moreover, certain mutations at non-cleavage sites in Gag have been shown previously to be essential for the replication of HIV-1 variants in the presence of PIs (14, 15). Although a few amino acid substitutions at cleavage and non-cleavage sites in Gag have been shown to be associated with resistance to PIs, the roles and impact of amino acid substitutions in Gag for the HIV-1 acquisition of PI resistance remain to be elucidated.

In the present study, we identified novel Gag non-cleavage site mutations in addition to multiple mutations in the protease gene during in vitro selection of HIV-1 variants highly resistant to amprenavir (APV). We show that the non-cleavage site mutations were important for not only the replication of the mutated-protease-carrying HIV-1 but also the accelerated acquisition of HIV-1 resistance to APV and an unrelated PI, nelfinavir (NFV). We also show that recombinant HIV-1 clones containing NFV resistance-associated mutations, such

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as D30N and N88S, had increased susceptibility to APV, suggesting that antiretroviral regimens including both APV and NFV may bring about favorable antiviral efficacy.

MATERIALS AND METHODS

Cells and antiviral agents. MT-2 and MT-4 cells were grown in RPMI 1640-based culture medium, and 293T cells were propagated in Dulbecco's modified Eagle's medium. These media were supplemented with 10% fetal calf serum (HyClone, Logan, UT), 50 U/ml penicillin, and 50 μg/ml streptomycin. APV was kindly provided by GlaxoSmithKline, Research Triangle Park, NC. Saquinavir (SQV) and ritonavir (RTV) were provided by Roche Products Ltd. (Welwyn Garden City, United Kingdom) and Abbott Laboratories (Abbott Park, IL), respectively. NFV and indinavir (IDV) were kindly provided by Japan Energy Inc., Tokyo.

Generation of PI-resistant HIV-1 in vitro. For the generation of PI-resistant HIV-1, various PI-resistant HIV-1 strains were propagated in the presence of increasing concentrations of a drug in a cell-free fashion as described previously (44, 45). In brief, on the first passage, MT-2 or MT-4 cells (5×10^5) were exposed to 500 50% tissue culture infective doses ($TCID_{50}$) of each infectious molecular HIV-1 clone and cultured in the presence of various PIs at initial concentrations of 0.01 to 0.06 μM. On the last day of each passage (approximately day 7), 1 ml of the cell-free supernatant was harvested and transferred to a culture of fresh uninfected cells in the presence of increased concentrations of the drug for the following round of culture. In this round of culture, three drug concentrations (increased by one-, two-, and threefold compared to the previous concentration) were employed. When the replication of HIV-1 in the culture was confirmed by substantial Gag protein production (greater than 200 ng/ml), the highest drug concentration among the three concentrations was used to continue the selection (for the next round of culture). This protocol was repetitively used until the drug concentration reached the targeted concentration. Proviral DNA from the lysates of infected cells at various passages was subjected to nucleotide sequencing.

Determination of nucleotide sequences. Molecular cloning and the determination of nucleotide sequences of HIV-1 passaged in the presence of each PI were performed as described previously (44, 45). In brief, high-molecular-weight DNA was extracted from HIV-1-infected MT-2 and MT-4 cells by using the InstaGene matrix (Bio-Rad Laboratories, Hercules, CA) and was subjected to molecular cloning, followed by sequence determination. The primers used for the first-round PCR amplification of the entire Gag- and protease-encoding regions of the HIV-1 genome were LTR F1 (5′-GAT GCT ACA TAT AAG CAG CTG C-3′) and PR12 (5′-CTC GTG ACA AAT TTC TAC TAA TGC-3′). The first-round PCR mixture consisted of 5 μl of proviral DNA solution, 2.0 U of premix *Taq* (Ex *Taq* version; Takara Bio Inc., Otsu, Japan), and 12.5 pmol of each of the first-round PCR primers in a total volume of 50 μl. The PCR conditions used were an initial 2-min step at 94°C, followed by 30 cycles of 30 s at 94°C, 30 s at 58°C, and 3 min at 72°C, with a final 8 min of extension at 72°C. The first-round PCR products (1 μl) were used directly in the second round of PCR with primers LTR F2 (5′-GAG ACT CTG GTA ACT AGA GAT C-3′) and Ksma2.1 (5′-CCA TCC CGG GCT TTA ATT TTA CTG GTA C-3′) under the same PCR conditions described above. The second-round PCR products were purified with spin columns (MicroSpin S-400 HR; Amersham Biosciences Corp., Piscataway, NJ), cloned directly, and subjected to sequencing with a model 377 automated DNA sequencer (Applied Biosystems, Foster City, CA).

Generation of recombinant HIV-1 clones. The PCR products obtained as described above were digested with two of the three enzymes BssHII, ApaI, and SmaI, and the obtained fragments were introduced into $pHIV\text{-}1_{NLSma}$, designed to have a SmaI site by changing two nucleotides (2590 and 2593) of $pHIV\text{-}1_{NL4\text{-}3}$ (15, 19). To generate HIV-1 clones carrying the mutations, site-directed mutagenesis using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was performed, and the mutation-containing genomic fragments were introduced into $pHIV\text{-}1_{NLSma}$. Determination of the nucleotide sequences of plasmids confirmed that each clone had the desired mutations but no unintended mutations. 293T cells were transfected with each recombinant plasmid by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA), and the thus-obtained infectious virions were harvested 48 h after transfection and stored at −80°C until use.

Drug sensitivity assays. Assays for HIV-1 p24 Gag protein production were performed with MT-4 cells as described previously (1, 20, 24). In brief, MT-4 cells (10^5/ml) were exposed to 100 $TCID_{50}$ of infectious molecular HIV-1 clones in the presence or absence of various concentrations of drugs and were incubated at 37°C. On day 7 of culture, the supernatant was harvested and the amounts of p24 Gag protein were determined by using a fully automated chemiluminescent enzyme immunoassay system (Lumipulse F; Fujirebio Inc., Tokyo). The drug concentrations that suppressed the production of p24 Gag protein by 50% (50% inhibitory concentrations [IC_{50}]) were determined by comparing the levels of p24 production with that in a drug-free control cell culture. All assays were performed in triplicate.

Replication kinetic assay. MT-2 or MT-4 cells (10^5) were exposed to each infectious HIV-1 clone (5 ng of p24 Gag protein/ml) for 3 h, washed twice with phosphate-buffered saline, and cultured in 10 ml of complete medium as described previously (1, 14). Culture supernatants (50 μl) were harvested every other day, and the p24 Gag amounts were determined as described above.

CHRA. Two titrated infectious clones to be compared for their replicative capabilities or fitness in the competitive HIV-1 replication assay (CHRA) were combined and added to freshly prepared MT-4 cells (2×10^5) in the presence or absence of various concentrations of PIs as described previously (21, 36). Briefly, a fixed amount (200 $TCID_{50}$) of one infectious clone was combined with three different amounts (100, 200, and 300 $TCID_{50}$) of the other infectious clone, and the mixture was added to the culture of MT-4 cells. On the following day, one-third of infected MT-4 cells were harvested and washed twice with phosphate-buffered saline, and cellular DNA was extracted and subjected to nested PCR and sequencing as described above. The HIV-1 coculture that best approximated a 50:50 mixture on day 1 was further propagated, and the remaining cultures were discarded. Every 7 days, the cell-free supernatant of the virus coculture was transmitted to fresh uninfected MT-4 cells. The cells harvested at the end of each passage were subjected to direct DNA sequencing, and viral population changes were determined. The persistence of the original amino acid substitutions was confirmed for all infectious clones used in this assay.

Statistical analysis of selection profiles of infectious HIV-1 clones. The selection profiles of various infectious HIV-1 clones were compared as follows. The logarithms of the concentrations were modeled as normally distributed variables with possible left censoring. The mean was assumed to be a quadratic function of the passage number. The difference between two curves was assessed by combining the estimated covariance-weighted differences of the linear and quadratic coefficients and comparing the result to computer simulations for the same quantity generated under the specific null hypothesis for that difference. SAS 9.1.3 (SAS Institute, Cary, NC) was used for all the computations. All *P* values are two tailed, and for figures with more than two curves, the values were corrected by the Hochberg method for multiple pairwise comparisons.

RESULTS

Amino acid sequences of Gag and protease of HIV-1 passaged in the presence of APV. A wild-type HIV-1 strain (HIV_{WT}) was propagated in MT-2 cells in the presence of increasing concentrations of APV, and the proviral DNA sequences in those MT-2 cells were determined at passages 3, 12, and 33 (Fig. 1). By passage 3, when HIV-1 was propagating in the presence of 0.04 μM APV (yielding HIV_{APVp3}), no amino acid substitutions in protease were identified but 5 of 10 clones had acquired the substitution of arginine for leucine at position 75 (L75R) in Gag. By passage 12 (at 0.18 μM APV), two APV-related resistance mutations (L10F and M46L) in protease had emerged and one mutation (H219Q) in Gag had been added. By passage 33 (at 10 μM; yielding HIV_{APVp33}), six APV-related amino acid substitutions, one primary mutation (I84V) and five secondary mutations (L10F, V32I, M46I, I54M, and A71V), in protease had emerged (Fig. 1A). In addition, a p1-p6 cleavage site mutation in Gag (L449F) was identified in all 10 HIV-1 clones of HIV_{APVp33} examined, and five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K) were seen in Gag of HIV_{APVp33} (Fig. 1B). Cleavage site mutations have been known to emerge when amino acid substitutions in protease are accumulated and HIV-1 develops resistance to PIs both in vitro and in vivo (5, 8). Intriguingly, the present data suggest that certain amino acid substitutions in non-cleavage sites of Gag (i.e., L75R and

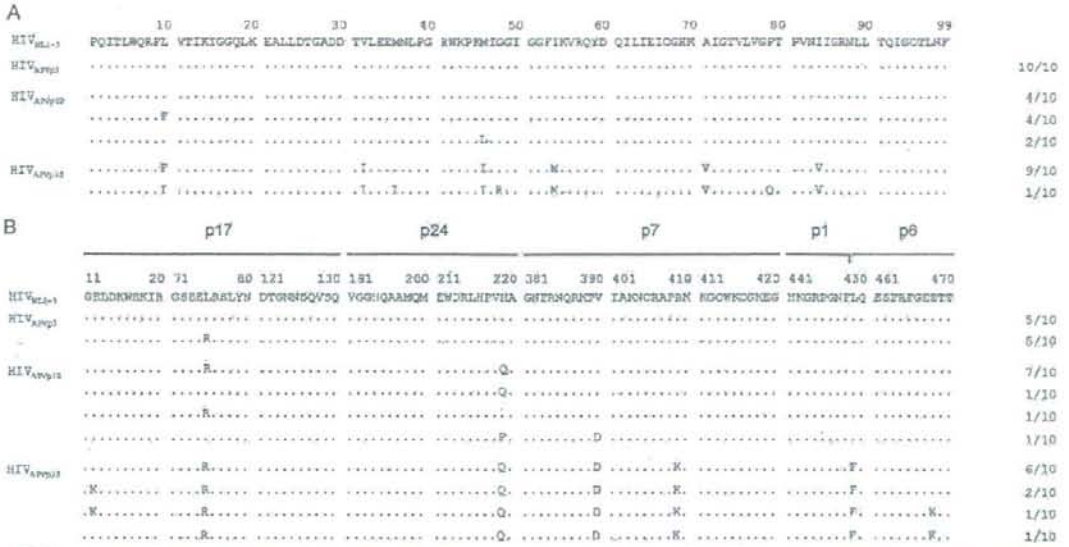

FIG. 1. Amino acid sequences deduced from the nucleotide sequences of protease (A)- and Gag (B)-encoding regions of proviral DNA isolated at the indicated passages (p3, p12, and p33) from HIV-1_{NL4-3} variants selected in the presence of APV. The amino acid sequences of the protease and Gag proteins of wild-type HIV-1_{NL4-3} are shown at the top as a reference. Identity to the sequence at individual amino acid positions is indicated by dots. The numbers of clones with the given amino acid substitutions among a total of 10 clones are listed.

H219Q) may emerge earlier and in greater numbers than amino acid substitutions in protease, at least in the case of HIV-1 selection with APV. The amino acid substitutions that emerged in the virus and the pattern and order of such substitutions were largely in agreement with the data in the previous report by Gatanaga et al. (15). The present results suggested that the non-cleavage site mutations observed may play a key role in the development of HIV-1 resistance against PIs and that especially the two Gag mutations H219Q and R409K may be required for the development of PI resistance.

Mutations in Gag are required for the replication of HIV_{APVp33}. In order to examine the effects of the mutations identified in Gag as described above on the replication profile of HIV-1, we generated infectious recombinant HIV-1 clones containing the six mutations (L10F, V32I, M46I, I54V, A71V, and I84V) in protease seen in HIV_{APVp33}. A recombinant HIV-1 clone containing the protease of HIV_{APVp33} plus a wild-type Gag ($rHIV_{APVp33pro}^{WTgag}$) or the L449F cleavage site mutation-containing Gag ($rHIV_{APVp33pro}^{449gag}$) failed to replicate in MT-2 cells over the 7-day period of culture (Fig. 2A), indicating that these Gag species do not support the growth of HIV_{APVp33}. Therefore, we next generated a recombinant HIV-1 clone containing the protease of HIV_{APVp33} and the Gag protein with the five non-cleavage site mutations (E12K, L75R, H219Q, V390D, and R409K; $rHIV_{APVp33pro}^{12/75/219/390/409gag}$), which replicated moderately under the same conditions (Fig. 2A). The addition of the cleavage site mutation L449F, generating $rHIV_{APVp33pro}^{12/75/219/390/409/449gag}$, further improved the replication of the virus. In MT-4 cells, in which HIV-1 generally replicates more quickly and efficiently than in MT-2 cells, $rHIV_{APVp33pro}^{WTgag}$ and $rHIV_{APVp33pro}^{449gag}$ replicated moderately; however, both $rHIV_{APVp33pro}^{12/75/219/390/409gag}$ and $rHIV_{APVp33pro}^{12/75/219/390/409/449gag}$ replicated comparably to HIV_{WT} (Fig. 2B), due presumably to the greater replication of HIV-1 in MT-4 cells, making the difference relatively indistinct. These data clearly indicate that both non-cleavage site and cleavage site mutations in Gag contribute to the robust fitness of HIV_{APVp33}. We also attempted to examine the effects of combined Gag mutations on the replication of HIV-1 containing wild-type protease and generated three recombinant HIV clones, $rHIV_{WTpro}^{75/219gag}$, $rHIV_{WTpro}^{219/409gag}$, and $rHIV_{WTpro}^{12/75/219/390/409gag}$. The replication rates of these three recombinant clones turned out to be comparable to that of HIV_{WT} when examined in MT-2 and MT-4 cells (Fig. 2C and D), unlike the finding by Doyon and his colleagues that the cleavage site mutation L449F compromised the replication of HIV-1 containing wild-type protease (8).

Gag mutations predispose HIV-1 to rapidly acquire APV resistance. The appearance of two non-cleavage site mutations (L75R and H219Q) in Gag prior to the emergence of mutations in protease (Fig. 1) prompted us to examine whether these two Gag mutations predisposed the virus to the acquisition of APV resistance-associated mutations in protease. We thus attempted to select APV-resistant HIV-1 by propagating HIV_{NL4-3} (HIV_{WT}) and $rHIV_{WTpro}^{75/219gag}$ in the presence of increasing concentrations of APV (Fig. 3). When we compared the selection curves of these two viruses, there was no significant difference (P, 0.53 and 0.65 for propagation in MT-2 and MT-4 cells, respectively). We then examined the effects of two mutated Gag species containing two and five mutations (H219Q and R409K and E12K, L75R, H219Q, V390D, and

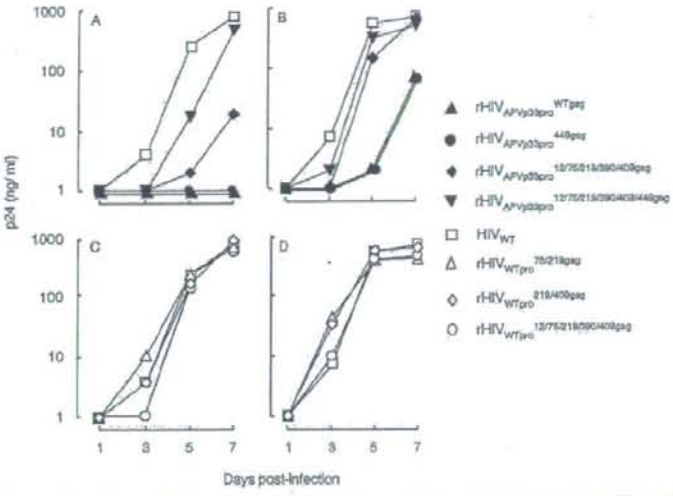

FIG. 2. Replication kinetics of Gag mutant clones with or without protease mutations. MT-2 cells (A and C) and MT-4 cells (B and D) were exposed to Gag mutant clones with (A and B) or without (C and D) protease mutations. Virus replication was monitored by the amounts of p24 Gag produced in the culture supernatants. The results shown are representative of results from three independent experiments. HIV$_{APVp33}$ variants had six mutations (L10F, V32I, M46I, I54M, A71V, and I84V) in the viral protease.

R409K [yielding mGag$^{12/75/219/390/409gag}$], respectively) on the selection curves. The selection profile of a newly generated recombinant HIV clone (rHIV$_{WTpro}^{219/409gag}$) was not different from that of HIV$_{WT}$ in MT-2 cells ($P = 0.22$); however,

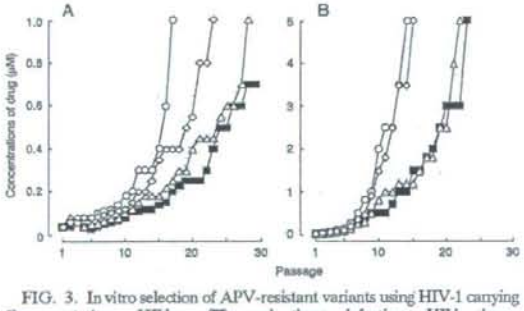

FIG. 3. In vitro selection of APV-resistant variants using HIV-1 carrying Gag mutations. HIV$_{WT}$ (■) and three infectious HIV clones, rHIV$_{WTpro}^{75/219gag}$ (△), rHIV$_{WTpro}^{219/409gag}$ (◇), and rHIV$_{WTpro}^{12/75/219/390/409gag}$ (○), were propagated in the presence of increasing concentrations of APV (starting at 0.03 μM) in MT-2 cells (A) or MT-4 cells (B). The selection was carried out in a cell-free manner for a total of 14 to 29 passages. The results of statistical evaluation of the selection profiles are as follows: panel A, HIV$_{WT}$ versus rHIV$_{WTpro}^{75/219gag}$, $P = 0.53$; HIV$_{WT}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P = 0.0080$; HIV$_{WT}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.22$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P = 0.0065$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.15$; and rHIV$_{WTpro}^{12/75/219/390/409gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.0018$, and panel B, HIV$_{WT}$ versus rHIV$_{WTpro}^{75/219gag}$, $P = 0.65$; HIV$_{WT}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P < 0.0001$; HIV$_{WT}$ versus rHIV$_{WTpro}^{219/409gag}$, $P < 0.0001$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{12/75/219/390/409gag}$, $P < 0.0001$; rHIV$_{WTpro}^{75/219gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P < 0.0001$; and rHIV$_{WTpro}^{12/75/219/390/409gag}$ versus rHIV$_{WTpro}^{219/409gag}$, $P = 0.088$.

rHIV$_{WTpro}^{219/409gag}$ acquired resistance to APV much earlier than HIV$_{WT}$ when propagated in MT-4 cells ($P < 0.0001$). The recombinant clone with five non-cleavage site mutations (rHIV$_{WTpro}^{12/75/219/390/409gag}$) started to propagate in both cell lines in the presence of APV significantly earlier than HIV$_{WT}$, with P values of 0.0080 and <0.0001 for MT-2 and MT-4 cells, respectively (Fig. 3).

We then asked whether additional amino acid substitutions occurred and accelerated the acquisition of APV resistance by the virus when the Gag mutations were present. To investigate this issue, we determined the nucleotide sequence of the protease-encoding gene of each virus. Only one protease mutation (L10F) was seen by passage 20 when HIV$_{WT}$ and rHIV$_{WTpro}^{75/219gag}$ were propagated in MT-2 cells in the presence of APV (Fig. 4A and B). In contrast, two mutations (M46L and I84V) had been acquired by rHIV$_{WTpro}^{219/409gag}$ by passage 20. Of note, when rHIV$_{WTpro}^{12/75/219/390/409gag}$ was propagated in MT-2 cells in the presence of APV, a mutation (L10F) had occurred by an early passage (passage 5) and four mutations (L10F, V32I, M46I, and I84V) had emerged by passage 17 (Fig. 4D). When examined in MT-4 cells, HIV$_{WT}$ and rHIV$_{WTpro}^{75/219gag}$ had acquired two mutations (L10F and I84V and M46L and I84V, respectively) by passage 10, although rHIV$_{WTpro}^{219/409gag}$ and rHIV$_{WTpro}^{12/75/219/390/409gag}$ had acquired three and four mutations (L10F, M46I, and I84V and L10F, V32I, M46I, and I84V, respectively) by the same passage (Fig. 4E to H). These data, taken together, indicate that the two sets of Gag mutations (H219Q and R409K and E12K, L75R, H219Q, V390D, and R409K) clearly predisposed the virus to rapidly acquire APV resistance-associated mutations in protease and begin to propagate in the presence of APV.

Gag mutations in HIV$_{APVp33}$ delay viral acquisition of resistance to other PIs. We next asked whether the presence of

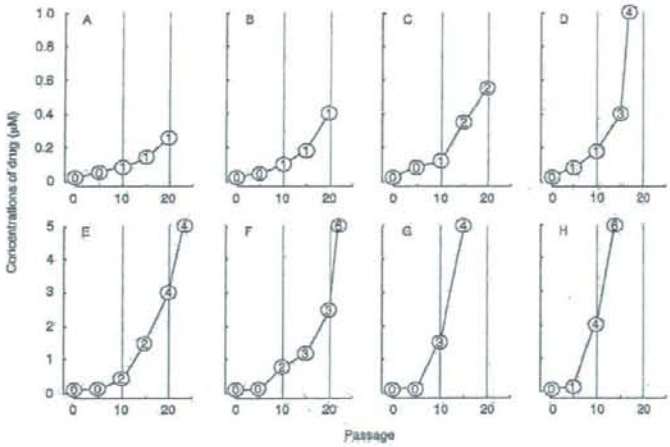

FIG. 4. Number of amino acid substitutions corresponding to the protease-encoding region of each infectious HIV-1 clone selected in the presence of APV. Nucleotide sequences of proviral DNA of HIV_{WT} (A and E) and three infectious HIV-1 clones, $rHIV_{WTpro}^{75/219gag}$ (B and F), $rHIV_{WTpro}^{219/409gag}$ (C and G), and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (D and H), were determined using lysates of HIV-1-infected MT-2 cells (A to D) and MT-4 cells (E to H) at the termination of each passage and compared to the nucleotide sequence of $HIV\text{-}1_{NL4\text{-}3}$. The number within each symbol represents the number of mutations identified in the protease when each infectious HIV-1 clone was selected in the presence of APV.

the five Gag mutations (E12K, L75R, H219Q, V390D, and R409K) accelerated the viral acquisition of resistance to other currently available PIs (SQV, IDV, RTV, and NFV) (Fig. 5). To this end, we propagated two HIV-1 strains (HIV_{WT} and $rHIV_{WTpro}^{12/75/219/390/409gag}$) in MT-4 cells in the presence of increasing concentrations of each PI and compared the replication profiles. The initial drug concentrations used were 0.01 μM for SQV, 0.03 μM for IDV and NFV, and 0.06 μM for RTV, and each virus was selected by a concentration of up to 5 μM. The selection was carried out in a cell-free manner for a total of 13 to 32 passages as described previously (44, 45). There was no significant difference in the selection profiles of the two strains when they were passaged in the presence of SQV ($P = 0.8$) or IDV ($P = 0.22$) (Fig. 5A and B). However, $rHIV_{WTpro}^{12/75/219/390/409gag}$ started to replicate significantly later in the presence of RTV ($P = 0.0001$ (Fig. 5C). The selection profiles of HIV_{WT} and $rHIV_{WTpro}^{12/75/219/390/409gag}$ in the presence of NFV were examined in two independent experiments. Both curves in the first and second sets depicted in Fig. 5D showed statistically significant difference, with P values of <0.0001 and 0.0016, respectively. These data strongly suggest that the Gag mutations seen in HIV_{APVp33} predispose HIV-1 to the rapid acquisition of APV resistance; however, such Gag mutations delay the viral acquisition of resistance to other PIs.

Gag mutations seen in HIV_{APVp33} do not affect viral susceptibilities to PIs. Since the Gag mutations seen in HIV_{APVp33} were found to contribute to the rapid acquisition of viral resistance to APV but they delayed the emergence of viral resistance to other PIs, we examined whether such Gag mutations affected the susceptibilities of HIV-1 to various PIs in the HIV-1 drug susceptibility assay. As shown in Table 1, none of three sets of Gag mutations, as examined in the context of $rHIV_{WTpro}^{75/219gag}$, $rHIV_{WTpro}^{219/409gag}$, and

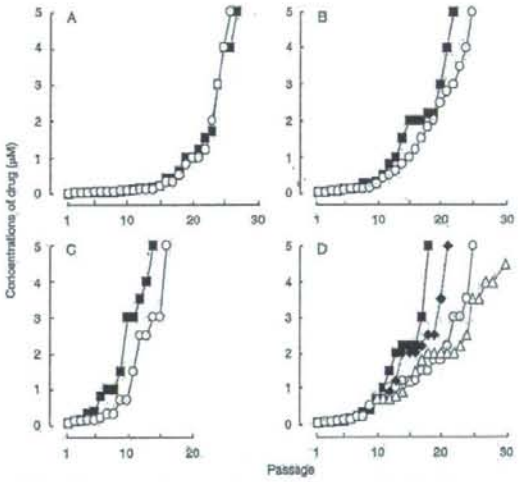

FIG. 5. In vitro selection of PI-resistant variants using HIV-1 carrying Gag mutations. HIV_{WT} (■ and ◆) and $rHIV_{WTpro}^{12/75/219/390/409gag}$ (○ and △) were propagated in MT-4 cells in the presence of increasing concentrations of SQV (A), IDV (B), RTV (C), or NFV (D). The initial drug concentrations used were 0.01 μM for SQV, 0.03 μM for IDV and NFV, and 0.06 μM for RTV, and each virus was selected by up to a 5 μM concentration of each PI. The selection was carried out in a cell-free manner for a total of 13 to 32 passages. NFV selection was performed twice. Data from the first selection are shown with a solid line; the second selection was started using the HIV-1 from passage 10 of the first selection (with NFV at 0.7 μM), and the data are shown with a dashed line. The results of statistical evaluation of the selection profiles are as follows: panel A, $P = 0.80$; panel B, $P = 0.22$; panel C, $P = 0.0001$; and panel D, first selection, $P < 0.0001$, and second selection, $P = 0.0016$.