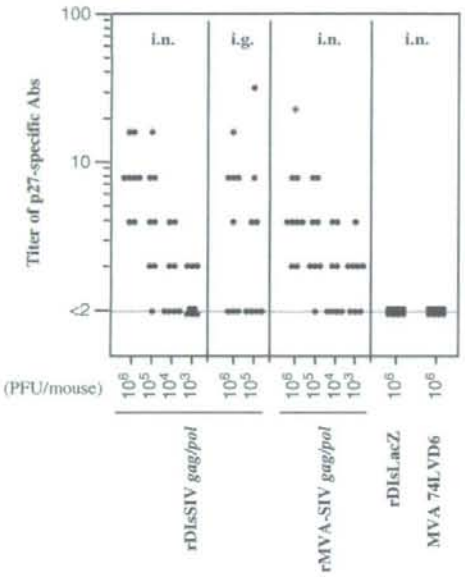

Figure 1 Titres of SIV p27-specific IgA Ab in fecal extracts of mice immunized with rDIsSIV*gag/pol* or rMVA-SIV*gag/pol*. Fecal pellets were collected at 1 week after the last immunization. The titres of p27-specific IgA Ab in the fecal extracts of mice immunized with rDIsSIV*gag/pol* or rMVA-SIV*gag/pol* were determined using an endpoint ELISA. The data are representative of two separate experiments. Each group was compared by a two-tailed Mann–Whitney *U*-test. Significant differences between rDIs group and rMVA group are indicated by asterisks (*$P < 0.05$). in, intranasal; ig, intragastrical.

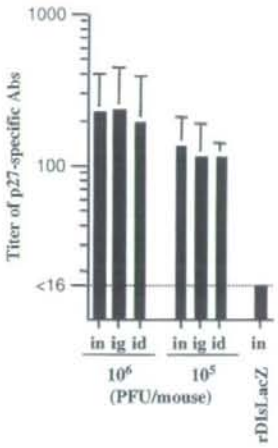

Figure 2 Comparison of SIV p27-specific IgG Ab responses in the plasma of mice immunized via different routes. Plasma samples were collected at 1 week after the last immunization. The titres of p27-specific IgG Ab in the plasma of mice immunized with 10^5 or 10^6 PFU of rDIsSIV*gag/pol* were determined using an endpoint ELISA. The data are shown as the mean titre + SD for 12 mice in each experimental group. Data are representative of three separate experiments. in, intranasal; ig, intragastrical; id, intradermal.

Induction of SIV p27-specific Ab in plasma of mice immunized via mucosal routes

Our previous studies showed that SIV Gag-specific immune responses were induced in mice intradermally immunized with rDIsSIV*gag/pol* [12]. We assessed that IgG Ab response to SIV p27 in the plasma of immunized mice at 1 week after the last immunization. Our results clearly show that intranasal, intragastrical, and intradermal immunization with rDIsSIV*gag/pol* induced p27-specific IgG Ab in plasma (Fig. 2), with similar titres of p27-specific IgG observed for intranasal, intragastrical and intradermal groups receiving the same dose. In contrast, the titres of < 16 were observed for p27-specific IgG Ab in plasma of control mice immunized intranasally with 10^6 PFU rDIsLacZ.

Induction of p27-specific AFC in the mucosal and systemic immune systems

Once the SIV p27-specific Ab responses had been further confirmed at the cellular level, we compared the number of p27-specific AFC induced in the mucosal and systemic lymphoid tissues after intranasal and intragastrical immunization with 10^5 PFU of rDIsSIV*gag/pol*. When we quantitated p27-specific AFC in the mucosal tissues and spleens of mucosally immunized mice, we found clear evidence of p27-specific IgA AFC in the PP, i-LP, NALT and n-LP of mice immunized intranasally with 10^5 PFU of rDIsSIV*gag/pol* (Fig. 3A). The numbers of p27-specific IgA AFC in the i-LP of intragastrically immunized mice were significantly higher than in intranasally immunized mice. Conversely, intranasal immunization of rDIsSIV*gag/pol* strongly induced p27-specific IgA AFC in the NALT and n-LP. We also found the number of p27-specific IgG AFC in the spleen of mice immunized intranasally with rDIsSIV*gag/pol* to be significantly higher than in intragastrically immunized mice ($P < 0.05$) (Fig. 3B). These findings suggest that mucosally administered rDIs can act as a vector for the induction of p27-specific AFC in both mucosal and systemic tissues.

Helper cytokine profiles of SIV Gag-specific CD4$^+$ T cells

Simian immunodeficiency virus Gag-specific helper T-cell responses were assessed using cytokine-specific ELISA for culture supernatants of CD4$^+$ T cells isolated from the spleen of immunized mice. Our results demonstrated that both type 1 helper T cell (Th1) and Th2 cytokines were upregulated in overlapping Gag peptide-stimulated CD4$^+$ T cells taken from the spleen of mice immunized with 10^5 PFU of rDIsSIV*gag/pol*. Of special note, the levels of IFN-γ in mice immunized intranasally with

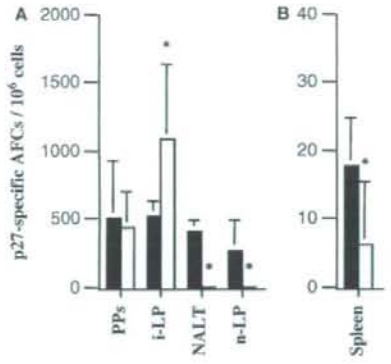

Figure 3 SIV p27-specific IgA AFC in the mucosal tissues and IgG AFC in systemic tissues of mice immunized intranasally or intragastrically with rDIsSIV*gag/pol*. The mice were killed 1 week after the last immunization. Levels of p27-specific IgA AFC in PP, i-LP, NALT and n-LP (A) and levels of p27-specific IgG AFC in spleen (B) of mice immunized intranasally (closed column) or intragastrically (open column) with 10^5 PFU rDIsSIV*gag/pol* were determined using an ELISPOT assay. The data are shown as the mean number of AFC/10^6 cells + SD for 12 mice in each experimental group. Data are representative of three separate experiments. Each group was compared by a two-tailed Student's *t* test. Significant differences between the intranasal group and intragastric group are indicated by asterisks (*$P < 0.05$).

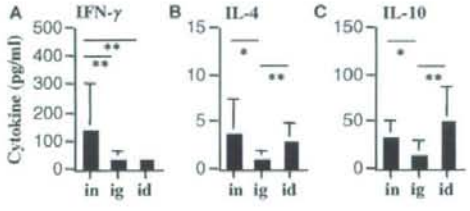

Figure 4 Th1/Th2 cytokine production of SIV Gag overlapping peptide-stimulated CD4+ T cells of mice immunized with 10^5 PFU rDIsSIV*gag/pol*. CD4+ T cells were isolated from spleen at 1 week after the last immunization. Culture supernatants were harvested and then analysed for the production of IFN-γ (A), IL-4 (B), and IL-10 (C) by ELISA. The levels of Gag-specific cytokine production were calculated by subtracting the results of the control culture from the peptide-stimulated culture. The data are shown as the mean concentration + SD for 12 mice in each experimental group. Data are representative of three separate experiments. Each group was compared by a two-tailed Student's *t* test. Significant differences are indicated by asterisks (*$P < 0.05$, **$P < 0.005$). in, intranasal; ig, intragastrical; id, intradermal.

rDIsSIV*gag/pol* were significantly higher than in those intragastrically or intradermally immunized (Fig. 4A). However, no preferential association was noted between Th2 and either the intranasal or intradermal group (Fig. 4B, 4C). In contrast, the intragastrical group showed significantly lower levels of Th2 cytokines than did the intranasal or intradermal group.

IFN-γ production of SIV Gag-specific CD8+ T cells

Cytotoxic T-lymphocyte activity in viral infection has been shown to be of central importance for host defence. To assess the mucosal induction of cellular immunity, we assessed the CD8+ IFN-γ -producing cells in the immunized mice. Because non-specific activated CD8+ cells produced IFN-γ, the number of SIV Gag-specific IFN-γ SFC was calculated (see *Materials and methods*). In the IEL of mice immunized intranasally with rDIsSIV*gag/pol*, the numbers of IFN-γ SFC in unstimulated condition were 70–460 cells/10^6 CD8+ cells. The numbers of IFN-γ SFC in Gag peptide-stimulated condition were 150–1385 cells/10^6 CD8+ cells. There were significant differences between stimulated and unstimulated group. It was demonstrated that SIV Gag-specific IFN-γ-producing CD8+ cells appeared in both systemic and mucosal compartments in the intranasally immunized mice and were particularly abundant in the IEL of the mice (Fig. 5).

Kinetics of p27-specific Ab in plasma

IgA, IgG and IgM class-specific endpoint ELISA was used to investigate an SIV p27-specific Ab in the plasma of mice immunized intranasally with 10^5 PFU of rDIsSIV*gag/pol*. Up until 11 weeks after the last immunization, the titres of p27-specific IgA Ab were low (mean values < 50). Without further boosting, increased p27-specific IgA Ab titres were observed after 11 weeks and were maintained in the plasma for approximately 1 year (49 weeks) at a mean value between 48 and 92 (Fig. 6A). Levels of p27-specific IgA Ab did not correlate with those of IgG and IgM Ab; p27-specific IgG Ab were maintained in the plasma for 1 year, and increases in IgG Ab titres were observed until 17–21 weeks after the last immunization. After peaking, the mean titre in

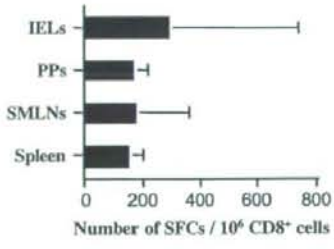

Figure 5 IFN-γ production of SIV Gag overlapping peptide-stimulated CD8+ cells isolated from mucosal and systemic components of mice immunized with rDIsSIV*gag/pol*. CD8+ cells were isolated from IEL, PP, SMLN and spleen at 1 week after the last immunization. IFN-γ production was assessed by ELISPOT assay. The number of Gag-specific IFN-γ SFC was calculated by subtracting the results of the control culture from those of the peptide-stimulated culture. The data are shown as the mean number of SFC + SD for 12 mice in each experimental group. Data are representative of three separate experiments.

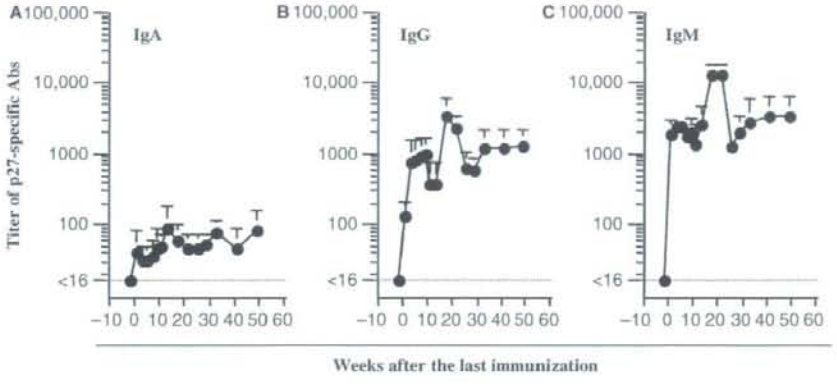

Figure 6 Sequential assessment of SIV p27-specific IgA, IgG, and IgM Ab in plasma of mice that have been intranasally immunized with 10^5 PFU rDIsSIV*gag/pol*. Plasma p27-specific IgA (A), IgG (B), and IgM (C) Ab were determined using an endpoint ELISA. The data are shown as the mean titres ± SD for 12 mice in each experimental group. Data are representative of three separate experiments.

intranasally immunized mice declined to a value between 580 and 1300 (Fig. 6B). The p27-specific IgM Ab were observed in the plasma of intranasally immunized mice throughout the 1-year period (Fig. 6C). Titre kinetics was similar for p27-specific IgM Ab and IgG Ab. Thus, it is important to note that the p27-specific plasma Ab responses were maintained for 1 year after the last immunization via the nasal route.

We quantitated p27-specific IgG, IgM and IgA AFC both in systemic tissues such as spleen and in mucosal tissues such as MLN, PP, and i-LP of the mice at 49 weeks after the last immunization. Our results clearly demonstrated that high numbers of p27-specific IgG, IgM and IgA AFC in mice immunized intranasally with 10^5 PFU of rDIsSIV*gag/pol* were maintained even for 1 year after immunization (Fig. 7). Moreover, the mean titres of p27-specific IgA Ab in fecal extracts from mice at 49 weeks were 54 ± 18 (n = 12).

Analysis of viral dissemination in the central nervous system

Adverse reactions to vaccinia virus can occur regardless of pre-existing susceptibilities [19]. Of the adverse events known to occur after smallpox vaccination [20, 21], the most serious is post-vaccinal encephalitis (PVE) [20]. Although the pathogenesis of PVE is unknown, vaccinia viruses were isolated from brain tissues in PVE cases [22, 23]. As the nasopharynx resides in close proximity to the brain, we sought to determine whether rDI is disseminated from the nasal cavity to the brain. The olfactory bulbs, cerebellum and cerebrum of mice immunized intranasally with 10^6 PFU of rDIsSIV*gag/pol* were assessed using both nested DNA PCR and RT-PCR. The SIV *gag* gene was not detected in the olfactory bulbs, cerebellum or cerebrum of any of the eight mice (data not shown).

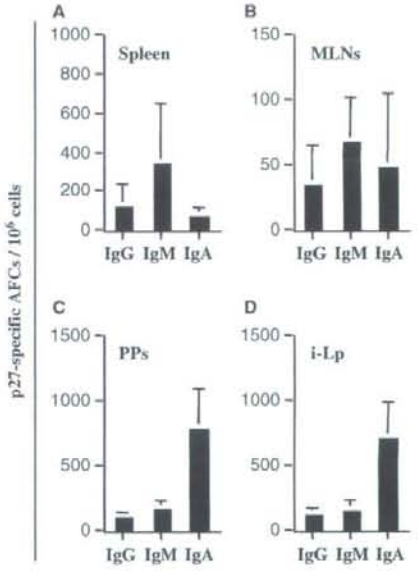

Figure 7 SIV p27-specific AFC in mucosal and systemic tissues of mice immunized intranasally with 10^5 PFU rDIsSIV*gag/pol* at 49 weeks after the last immunization. Levels of p27-specific IgG, IgM and IgA AFC in spleen (A), MLN (B), PP (C), and i-LP (D) of mice were determined using an ELISPOT assay. Data are shown as the mean number of AFC/10^6 cells ± SD for 12 mice in each experimental group and the data are representative of three separate experiments.

Discussion

In this study, p27-specific IgA Ab in fecal extracts and IgG Ab in plasma were confirmed in mice immunized intranasally or intragastrically with rDIsSIV*gag/pol*. The compartmentalization within the mucosal immune system places constrains on the choice of vaccination route

for inducing effective immune responses. Oral immunization induces substantial Ab responses in the small intestine [24, 25]. We also found that the numbers of p27-specific IgA AFC were strongly induced in the small intestine by intragastrical route. Conversely, intranasal immunization results in Ab responses in the upper airway mucosa and regional secretions without evoking an immune response in the gut [26, 27]. Although the numbers of p27-specific IgA AFC in i-LP of mice intranasally immunized rDIsSIV*gag/pol* were significantly lower than intragastrical group, the titres of p27-specific IgA Ab in fecal extracts of mice immunized intranasally or intragastrically were roughly equivalent. Intranasal immunization of rDIsSIV*gag/pol* dominantly induced p27-specific IgA AFC in nasal tissues, and these cells might powerfully produce dimeric or polymeric IgA Ab. Polymeric IgA Ab removed from the circulation into bile by the hepatic polymeric immunoglobulin receptor/secretory component-driven. A large portion of murine gut IgA Ab is derived from blood rather than mucosal production [28–30]. Taken together, this study showed that intranasal immunization of rDIs, induced not only potent plasma IgA Ab response but also a good intestinal IgA Ab level despite a poorer cellular IgA Ab response in intestinal mucosa.

One of the keys to vaccine development is the longevity and stability of immune memory. We have shown that p27-specific IgG, IgM and IgA Ab and p27-specific AFC remain detectable for at least 1 year after intranasal administration of replication-deficient rDIsSIV*gag/pol*. Interestingly, IgM Ab against p27 in plasma were detected throughout the period of Ab monitoring, and significant numbers of p27-specific IgM AFC were observed in spleen even 1 year after immunization. Significantly more p27-specific IgM AFC were observed in spleens, suggesting that they may originate from those central lymphoid tissues in immunized mice. Based on the results of the current study, we theorize that SIV Gag continued to be produced in the mice in sufficient quantities to reactivate IgM Ab production in the absence of viral replication and reinfection. Others have shown that hepatitis B virus core Ag-specific or hepatitis C virus capsid Ag-specific IgM Ab are found in the plasma of individuals in the chronic phase of hepatitis B or C viral infection [31, 32]. We have not assessed long-term rDIs vector persistence in mucosal tissues, and we have not yet managed to elucidate the mechanism governing prolonged p27-specific Ab induction. However, we showed that rDIs were able to induce long-term immunity against an inserted foreign Ag in intranasally immunized mice.

Intranasal vaccination has caused unexpected complications. Although DIs vector did not detected in the brain of mouse in this study, it is not clear about the safety for human brain. Intranasal vaccination with rDIs may pose risks such as PVE. It must concern the possibility that nasal vaccines could enter the central nervous system, because of the anatomical proximity of the nasal cavity to the brain. On the other hand, although oral delivery has become an accepted route for administration of polio-vaccine, the gastrointestinal tract presents several formidable barriers to candidate vaccines. In general, oral immunization is relatively inefficient at evoking an IgA Ab response in the distal segments of the large intestine or female genital tract mucosa [24, 25]. Of special interest for possible vaccination against HIV and other sexually transmitted infections, not only intravaginal but also intranasal immunization has been found to give rise to substantial IgA and IgG Ab responses in human cervicovaginal mucosae [24, 27, 33]. This study demonstrated that both intranasal and intragastrical immunizations of rDIs induce Ag-specific mucosal immune responses. However, we could not settle which is the better strategy of vaccine administration to prevent HIV infection in this study.

Of course, much more intensive examination of the efficacy and safety of the mucosal rDIs vaccine are needed before use in humans. Nonetheless, in this study we demonstrated that it is an effective vector for the induction of Ag-specific mucosal immunity in the mucosally immunized mouse model, and represents an important step towards the development of an effective mucosal rDIs vaccine against HIV.

Acknowledgments

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第21回日本エイズ学会シンポジウム記録

シンポジウム14 「HIV母子感染予防対策の20年」—現在の医学的・社会的問題点とその対策—

The History of HIV Mother to Child Transmission Prevention in Japan —Medical and Social Problems Left until Today—

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シンポジウムの趣旨

塚原優己，関矢早苗

1987年，本邦で初めてHIV感染妊娠例に妊娠中から分娩後まで一貫した母子管理が行われた。現行の感染予防対策の原型が考案され母子感染も回避できた。以来20年，この間にHIV治療は格段の進歩を遂げたが，残念なことにわが国の感染者は増加の一途をたどっている。母子感染に関する対応にも改良が加えられ，母子感染はほぼ回避可能と考えられるまでに至った。

HIV感染は，世界中で流行している重篤な感染症である。それゆえ医療者の間でも，また一般社会のなかでも問題意識が高く，他の感染症とは異なった対応を必要とすることも多い。HIV母子感染にも，HIVに特化した対応や，殊更細やかな配慮が求められている。そのなかには，既に通常の一般的な対応で十分と考えられるもの，未だに特別な配慮が必要なもの，どちらにすべきか意見が異なるものなどが混在している。

今回のシンポジウムの目的は，HIV母子感染に関わる様々な対応について，一般的な感染症と同等の標準的対応を目指す上での医学的・社会的問題点とその対策に焦点を当て，その妥当性を再検証することである。

妊婦HIVスクリーニング検査は，その実施率は年々上昇し日本全国でほとんど（約95%）の妊婦が受検するようになったが，一方でその陽性的中率の低さから医療現場で結果陽性妊婦への対応に混乱が生じているケースも散見される。この問題を踏まえ，妊娠初期に行う他のスクリーニング検査（梅毒，B型肝炎，C型肝炎，風疹抗体価，血算，血糖など）同様の対応（検査項目を明記した紙面を妊婦に手渡すことで説明と同意を簡略化）について，その問題点と対策を提示していただいた。

院内感染対策の分野では，現在スタンダードプリコーションが一般化している。HIV感染者の受け入れは，スタンダードプリコーションに基く院内感染対策マニュアルに準拠することだけで可能か？　妊娠分娩管理では血液や体液曝露による感染の危険性が高い。この点も考慮した上で，感染管理の立場から，HIV母子感染予防対策マニュアルの妥当性について検証していただいた。

近年，妊婦血中ウイルス量が母子感染の主たるリスクファクターと考えられ，またHAARTにより血中ウイルス量を感度以下まで抑えることも可能となった。ウイルス量の低い妊婦では母子感染予防のための帝王切開術は不要との議論も噴出している。はたして現在のHIV感染妊婦管理で，経腟分娩時の母子感染の危険性はどこまで回避可能か？　これまでに集積されたわが国の経腟分娩症例の臨床経過や諸外国からの報告をもとに，わが国の医療事情に照合して検討していただいた。

1980～90年代にHIV感染妊婦から出生した児は，既に思春期を迎えている。現在小児HIV感染は少数ではある

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が，HIV 感染者数が増加し続ければ将来母子感染の増加も危惧される。感染小児では，告知の問題を始め，HAART の継続，入園・入学，性教育など成長・発達に伴う様々な医学的・社会的問題が山積している。感染小児の療育の現状とその問題点の整理をお願いした。感染を回避し得た小児では，家族（両親）の HIV 感染に関わる問題を取りあげ，その対応を検討していただいた。

性行動は日常の自然な営みであり，また女性感染者が妊娠・出産を望むこともごく自然の心理である。しかし感染者が性交渉による自然の妊娠を求めた時には，少なからずパートナーへ感染の危険を伴なうことになる。感染回避という医学的観点からは，人工授精による妊娠が勧められるが，日常の性交渉により妊娠された例も数多い。人工授精に対する女性感染者の思いにも配慮した上で，妊娠・出産に関する情報提供を行う医療者の望ましい姿勢などについて考察していただいた。

1. 妊婦 HIV スクリーニング検査：これまで～今～そして今後の検討

矢永由里子

今回は次の 3 点 ① 妊婦 HIV 検査の現在の実施状況 ② 検査の現状と課題 ③ 今後の方向性や提言に絞って発表を行なった。これらの項目をまとめて，発表内容を報告したい。

【妊婦 HIV 検査の現在の実施率と検査の課題】

これまでの研究班の調査では，全国の妊婦 HIV 検査実施率は平成 18 年度に 95.3% まで上がっている。ほぼ妊婦の一般検査に HIV 検査も組み込まれると言える。しかし，一方でスクリーニング検査の偽陽性が妊婦群においては非常に高く，陽性的中率が約 10% という問題も残されている。

【電話相談や調査結果から見えてきたもの】

平成 18 年にエイズ予防財団の電話相談に寄せられた検査に関する相談を分析すると，HIV 検査方法の問い合わせが 29%，検査の信頼性が 23%，受検後の動揺を強く訴える相談が 22% であった。

また，18 年度に和田グループで実施した「HIV スクリーニング検査で『陽性』・その後確認検査で『陰性』となった妊婦の実態調査」では，30 名の妊婦を確認検査のために担当したエイズ診療拠点病院や検査・相談機関から，検査の実際についての動向を得ることが出来た。その概要は次の通りである。

① 検査のスタート地点である実施機関の説明の適・不適は妊婦の状態や理解と関連していた。

② 受け入れ機関における妊婦の心理状態は，実施機関のスクリーニング検査の意味理解の有無，妊婦が理解できる説明や結果告知の有無，メンタル面への配慮の有無と関連していた。

電話相談と実態調査が示す共通課題として，妊婦 HIV スクリーニング検査は，他の HIV 検査同様に受検者（妊婦）の理解に沿った適切なインフォームドコンセント下での実施と対応の必要性が挙げられる。19 年 6 月末日には，厚生労働省より各自治体の主管や医師会，産婦人科学会に対し，妊婦対象のスクリーニング検査前後の説明・相談の重要性とプライバシー保護への配慮についての通達が行なわれた。

【課題に対する取り組み】

妊婦への情報提供として，現在二種類のちらしが作成されている（①妊婦全般用として HIV 検査理解を促すため：「あなた自身の健康と赤ちゃんの健やかな誕生のために」② HIV 感染が判明した妊婦を対象として：「妊婦 HIV スクリーニング検査で結果が陽性だった方へ」）。また，19 年度末ごろには，HIV 検査を妊婦に実施する医療従事者向けに，検査前準備～検査前説明～結果説明をステップごとに具体的に提示した「妊婦 HIV 一次検査（スクリーニング検査）実施マニュアル」パンフレットを作成・配布の予定である。妊婦検査における病診連携の整備も急がれるが，一地域で実践されている検査実施機関と確認検査や HIV 陽性だった場合の診療の受け入れ医療機関との連携も重要である。今回発表した連携は妊婦検査のために作られたものではなく，妊産婦診療全般の連携のために日常的な情報交換やスムーズな妊婦紹介の活動が土台となり，そこに妊婦検査における連携も検討されていったという経緯があり，今後の病診連携のあり方を示唆された活動と思われる。

【今後の方向性や提言】

今後もスクリーニング検査で偽陽性を経験する妊婦数が継続すると思われるので，検査推進と同時に，偽陽性対応についての対策も一層強化する必要があるだろう。現在，妊婦への情報提供のちらしも作成されているが，今後は外国人妊婦にも情報提供の機会を広げていくことが大切と思われる。

偽陽性の課題とともに今後予測される状況として，HIV 検査で「陽性」が確定する妊婦数の増加である。今の日本における HIV 感染の広がりを鑑みれば，妊婦群での感染増加も想定される。現在，財団の電話相談に寄せられる妊婦検査の相談から，スクリーニング検査で「陽性」判明した妊婦に，安易に「最終結果は陰性だから」と「陰性」のみを強調する対応もなされている機関もあるようである。今後，陽性妊婦に対応する可能性も踏まえ，紹介先の医療機関の確保や地域連携のモデル体制の展開や感染妊婦の出産と地域での生活の場の確保なども重要になってくるだろう。

シンポジウム当日は，妊婦に関する HIV のテーマを多

角的に検討できたが，Opt-Out Testing のあり方や妊婦検査，検査時の情報提供や相談をどこまで行なうかという点について意見が出されたが，各人の感想に留まっているものが多く，今後このような重要テーマについては，実態や当事者の状況を正確に把握しつつ，また海外で作成されたコンセプトはその基本となる考えや実際の応用の全体像を明確にしながら議論を深めていく必要があると考える。

2. 感染管理の視点でとらえた「HIV 母子感染予防対策マニュアル」

内山正子

「HIV 母子感染予防対策マニュアル第 4 版」の院内感染対策に関する事項について，医療現場における感染対策の現状を踏まえながら感染管理の視点で捉え，改善策について検討した。

全ての湿性生体物質を感染性があるものとみなすスタンダードプリコーション（以下「SP」とする）の概念は，HIV 感染症の出現によって提唱されたユニバーサルプリコーションが時代とともに改変されたものである。したがって，HIV 感染者の受け入れは SP に基づく院内感染対策マニュアルに準拠することだけで可能であるといえる。

第 4 版の母子感染マニュアルにおける感染対策の事項は，ほとんどが SP に準拠した内容であるが，一方で SP を逸脱していると考えられる内容が見受けられるのも事実である。具体的には，日常診療や帝王切開時の過剰な防護用具の着用や器具，環境の覆い，新生児の沐浴などが挙げられる。

HIV だけに特化した感染対策が行われる理由は，医療従事者が，SP によって HIV の感染予防が可能であることを十分に認識していないこと，医療従事者自身への感染に対する強い懸念などが挙げられる。さらに SP が施設内において全ての患者さんに標準的に実施されていないため，HIV 感染者だけに SP が実施されるという矛盾が生じている。また，不要と思われる事項でも「マニュアルに記載されているため念のため実施する」という施設もある。特に HIV 感染者の受け入れの経験がない，あるいは少ない施設においては，特別な対応となりやすい。

以上のような現状をふまえ，マニュアル改訂時には，極力 SP に準拠した内容に再編成し，特別な対策は，血液や体液の曝露を防止するための工夫点として，曝露のリスクの高い処置やケアに限って記載することを提案する。具体的には，日常診療や看護ケアにおいては，特別な対応は不要で，再生器材は，HIV に限らず，次に使用する際に感染が起こらないよう適切な消毒あるいは滅菌が施されるため，HIV だけに特化してディスポ製品を使用する必要はない。帝王切開時は，顔面も含め血液に曝露しやすいため，術者や直接介助の看護師などが安心して手術に臨むことを目的に服装や覆いなどを通常より厳重にしてもよいが，麻酔医や間接介助看護師のディスポガウンは皮膚や衣服への血液曝露が想定されなければ不要と考える。新生児の沐浴は，清拭だけで対応しても感染が発生しておらず，沐浴によって感染防御の効果が高まるという根拠もないことから，通常と同様に清拭の記載でよいと考える。

以上，HIV 感染者の受け入れは SP に基づく院内感染対策マニュアルに準拠するだけで可能であることから，母子感染マニュアルの改訂では，極力 SP に準拠した内容とし，感染者への対応だけにマニュアルを活用するのではなく，自施設の SP の実施状況の見直しに活用してほしい。最後に，全ての医療施設において SP の実施が徹底され，HIV 感染者への特別な対応がなくなることを期待する。

3. HIV 感染妊娠の自然（経腟）分娩

喜多恒和，稲葉憲之

近年欧米では，HIV 感染妊娠の経腟分娩は母子感染に関しても安全であるとして，これを推奨する傾向がある。われわれは，厚労省研究班の研究成果をもとに HIV 感染妊娠の分娩転帰を解析し，わが国における経腟分娩の妥当性について検討した。2006 年 2 月までに産婦人科・小児科全国調査にて報告された 468 例の HIV 感染妊娠のうち，母子感染が確認されたのは 42 例であった。その 42 例の内訳は選択的帝切分娩が 7 例（母子感染率 3.2%）で，帝切の適応は母子感染予防が 1 例のみで残りは産科的適応であり，緊急帝切分娩は 4 例（同 18.2%）で，すべて産科的適応による帝切であった。経腟分娩は 25 例（同 50%）であったが，感染診断や投薬などの予防対策が講じられていた例はなかった。分娩様式不明は 6 例であった。一方経腟分娩で母子感染しなかったのは 25 例で，妊娠中から感染が判明したため免疫機能の測定やウイルス量のコントロールが行われていた 5 例が含まれている。しかし母子感染例の大多数が児の発症などを機に母親の HIV 感染が判明した例であるため，各分娩法の母子感染率は高く正確な比較はできない。産婦人科のみの全国調査での母子感染率の比較が妥当と考えられ，この解析から母子感染率は選択的帝切 0.5%（1/194），緊急帝切 5.9%（1/17），経腟分娩 20.8%（5/24）となった。経腟分娩による母子感染の最終報告は 2000 年であり，その後の HAART 時代での報告はない。しかも HIV 感染妊婦に対する HAART を中心とする抗ウイルス療法は上記分娩法の 80%，53%，14% に行われており，母子感染率の差は分娩法と抗ウイルス療法の相加結果であると考えられる。HAART により血中ウイルス量がコントロールされている場合の経腟分娩の安全性は否定できない。2005 年 Cochrane Database の報告でも同様の結論を示

しているが，2005年ヨーロッパからの報告ではHAARTを行っていても選択的帝切のほうが経腟分娩より安全であるとしている。一方2006年インドからの報告では，AZT単独療法後でも選択的帝切と経腟の2つの分娩法の母子感染率に統計学的有意差はないとしているものの，それぞれの母子感染率は1.1%と2.1%で，経腟分娩の母子感染率はほぼ2倍であった。HIV感染妊婦に対しHAARTを行いウイルス量が良好にコントロールされている場合においては，妊婦とその家族へ各国の医療技術や医療経済のレベルと母子感染率に関する正確な情報を提供した後，インフォームド・チョイスにしたがって分娩法が選択されるべきであると考えられた。

4. HIV感染妊婦から出生した児の成長発達支援

外川正生，稲葉憲之，和田裕一

厚労科研稲葉班の全国病院小児科調査によれば，1984年から2006年の間にHIV感染女性から出生した児は287例であり，感染児は42例，非感染児は199例，未確定・不明は46例であった。母児への抗ウイルス治療・予定帝王切開・断乳による母子感染予防対策が全て実施された群での感染率は0.6%（同班産科調査では0.5%）まで減少した。しかし対策無し，または不完全が理由での母子感染例は2000年以降も現れている。免疫不全が乳児期早期に発症するとHAARTが適用できる現在でもAIDS発症または死亡の確率が高く，難病であることに変わりがない。HAARTが時宜を得て開始され免疫が回復した小児も，生涯にわたっての服薬が必要であり，耐性・副作用・アドヒアランスの課題が待ちかまえている。出生児の現状から，子どもたちの健やかな成長にとって何が必要かを考察した。

【非感染児の課題】

新生児への抗ウイルス療法は従来AZT単独であったが，2NRTIあるいはHAARTが選択される例も現れた。生後の貧血に対してはPCR陰性結果を勘案したAZT投与期間短縮例が増える傾向にあった。平均観察期間2年1ヵ月（2ヵ月～5年2ヵ月）の50例においては，ミトコンドリア機能障害に合致する報告は確認されなかったが，就学年齢までの発育発達を確認することと，親あるいは感染した同胞についての告知あるいは病状を受けとめるに際しての精神的支援を欠かしてはならないであろう。

【感染児の臨床】

42例の予後は，無症状23例（54.8%），中等症1例（2.4%），AIDS 3例（7.1%），死亡11例（26.2%），転帰不明4例（9.5%）であった。初診年齢が4歳未満の26例では，AIDSまたは死亡が11例（42%）と予後不良であったが，HAARTが実施された15例では12例が良好に経過していた。一方，初診時4歳以上の11例ではHAART未実施でも3例中2例が，さらにHAART実施群では8例中7例が良好に経過していた。休薬例での薬剤耐性出現有無については情報が得られなかった。

【水平感染予防】

本邦のHIV感染妊婦は1万人に1人，母子感染率は自然状態で30%強，対策実施で0.5%であるから，感染児の出現頻度は1/200万から1/3万である。現在0～18歳に分布する感染児の殆どがHAARTの恩恵によって健康に生活している。彼らからの水平感染は，標準的予防によって可能である。幼稚園・学校などの指導者が，血液を媒介する感染症の普遍的存在を認識して，出血の場面では全ての子どもに対して同じ方法で処置することが必要である。HBV・HCVキャリアー同様，HIV感染児は全ての学校行事に参加可能であり，病状公表を強制されてはならない。

【告知・カウンセリング】

病名告知は通院・検査・HAARTを正しく受容するために10歳頃から必要となるが，定まった最適年齢はなく，本人の能力や環境を見究めて医師and/or家族が判断するのが現状である。病名告知に到らなくても，病態の説明，周囲へのカウンセリングから徐々に準備が進められている。他の慢性疾患同様，医師では行き届かない支援については，看護師・薬剤師・MSW・カウンセラー・保健師・通訳などが担当している。経験施設が限られているので，支援の過程における課題とその解決の実際について，記録を残すことが重要と考えられる。

5. 女性HIV陽性者の妊娠・出産支援

大金美和

HIV感染症の治療法が未確立の時代には，予後が極めて不良という疾患の特徴から，当時の医療者は二次感染予防と妊娠回避の指導をする傾向にあり，また既にHIV感染が判明した女性のなかには妊娠・出産をあきらめていたケースも多々散見される。しかし，治療法の進歩によりHIV感染症の予後が著しく改善した昨今，感染者支援は長期療養における生活の質にその重点がおかれ，女性陽性者では自然の心理として妊娠・出産の希望も増えてきている。

挙児を希望しての性行為はパートナーへの感染の危険性を持ち合わせており，医療者は，この相反する問題に対応することが求められている。感染回避という観点では人工受精による妊娠も選択肢となる。ACC・ブロック拠点病院に通院中の女性HIV陽性者10人に聞き取り調査を実施したところ，その多くが挙児希望はあるものの人工授精に対しては抵抗感を持っていることが判明した。人工授精に対しては，「自然ではない」，「人工授精にかかる時間や通院の手間」，「経済的負担」，「パートナーへの精神的負担」等が抵抗を感じる主な理由であった。

女性 HIV 陽性者の中には，希望により自然妊娠し出産するケースや，望まない妊娠により人工妊娠中絶するケースがみられた。自然妊娠に関する問題として，パートナーへの感染の可能性，治療方針への影響などがあげられる。未治療の妊娠女性の場合には，免疫状態にかかわらず，母子感染予防としての抗 HIV 療法の開始が直ちに検討される。また治療中の妊娠女性でも，薬剤の組み合わせの見直しや器官形成期の服薬継続に関する判断などが突然求められることになる。このような突発事態を回避するためには，リプロダクティブヘルスと HIV 感染症の互いに密接に関連する問題を熟慮し，予め治療方針や将来設計を立てておくことが望ましい。つまり，陽性女性の治療と療養生活の安定のためには，支援者が妊娠前から妊娠・出産に関する意志決定過程に関わり，サポートすることが最も重要と考える。

偶発的な自然妊娠は，その後の転帰や療養生活にも影響を及ぼす。例えば，HIV 感染症の病態や治療に関する知識が乏しい場合には，療養生活の見通しが持てず，HIV 感染症への不安に加えて，妊娠や出産に対する漠然とした不安が生じる。また母子感染予防法や妊娠経過，出産に伴う母体と児の状態に関する情報不足もより不安を増強させる。あるいは，事前のパートナーとのコミュニケーション不足が原因で，妊娠後にお互いの妊娠・出産に関する考え方の違いが判明し，妊娠継続に関する女性自身の意志決定が揺らぐ場合もある。医療者の対応は，第一に通院早期から面接の機会を持つことが肝要であり，そのなかで現時点で行える医療とその限界についての情報を提供し相談を行いながら，女性自身の意志決定の過程を継続してサポートすることが求められる。具体的には，①HIV 感染症の病態や治療の概要の情報提供，②妊娠方法を含む家族計画の情報提供，③母子感染予防法や妊娠経過，出産に伴う母体と児の状態に関する情報提供，④夫婦それぞれに対して提供した情報の理解度の確認，および個別的な問題に対する助言，⑤パートナーや家族などからの支援体制の調整，⑥必要時にはカウンセリングなどの専門職を紹介し連携を図り支援，である。

これまで，妊娠している女性 HIV 陽性者に対する医療者の対応は，感染予防対策に焦点が置かれており，妊婦ケアというよりも HIV 感染症ケアに偏っている傾向が見られた。今後は，スタンダードプリコーションに基づき，過剰な予防対策を見直す必要もある。また，HIV 感染症特有のケアに取り組みながらも，妊娠女性に対し通常行っている一般的な妊婦ケアの充実にも目を向ける事が大切である。さらには，妊娠中のみならず，出産・育児にも喜びを持って日々過ごせることが大切であり，家族と母と子の愛着形成を促し，新しい家族形成を見守るための支援を行うことが課題となっている。

ま と め

この 20 年間，効果的な治療法である HAART の導入により，HIV 感染症はかつての不治の病から治療の継続が不可欠な慢性感染症に様変わりし，また社会からも性感染症のひとつとして正確に認識されるようになってきた。しかしながら，感染予防のキャンペーンは未だその効果が数字に表れず，わが国の感染者数は右肩上がりの増加を続ける現状にある。

母子感染に関しては，ほとんど全ての妊婦が通常の妊娠管理の一環として HIV スクリーニング検査を受検し，また母子感染予防対策では自然分娩やスタンダードプレコーションなど質の向上が検討され，社会医学的視点に立てば極めて好ましい方向に変化してきた。しかしこのような現代社会においても，慢性的に HAART を続ける陽性者の生活の質の向上を目指す上での支援策は，未だ十分とは言いがたい側面が指摘されている。陽性女性の妊娠・出産・育児は当然の課題としてクローズアップされ，陽性児では成長に伴い広がる社会活動に対する社会の受け入れ，あるいは陽性の親を抱える子供たちへの支援など，今後も感染者の増加が見込まれる現在，早急に対策を講じるべき課題は数多い。母子感染予防を考える上で，「妊婦 HIV スクリーニング検査の普及啓発と陽性妊婦に対する母子感染予防策の完遂」と「陽性が判明した感染女性とその家族および感染児への支援」は表裏一体の課題である。前者をほぼ達成しえた現在，これを維持しつつ取り組むべき次の課題は陽性者の支援と陽性者を取り巻く環境の整備ではないだろうか。

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Intracellular Efavirenz Levels in Peripheral Blood Mononuclear Cells from Human Immunodeficiency Virus-Infected Individuals▽

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We describe a novel method for isolating plasma-free peripheral blood mononuclear cells retaining intracellular efavirenz. Quantification of efavirenz in 13 human immunodeficiency virus-infected patients by liquid chromatography-tandem mass spectrometry showed a higher correlation of intracellular levels with unbound plasma levels (accumulation ratio, 1,190) than with total plasma levels.

Efavirenz, a nonnucleoside reverse transcriptase inhibitor, is a major component of current antiretroviral therapy for human immunodeficiency virus (HIV) type 1 infection. Several studies supported the idea of the usefulness of therapeutic drug monitoring of efavirenz, because viral suppression and adverse effects are associated with efavirenz levels in plasma (3, 9). Given that efavirenz exerts inhibitory activity with respect to reverse transcriptase and may interact with cellular enzymes within the cell, it is likely that intracellular levels are more relevant to the clinical outcome than plasma levels (10). Intracellular efavirenz levels have been explored using similar procedures, which consisted of preparation of peripheral blood mononuclear cells (PBMCs) by Ficoll density gradient centrifugation and cell washing with ice-cold phosphate buffered saline (PBS) (1, 2, 10). During our preliminary experiments, it was observed that efavirenz in PBS strongly stuck to the surface of plastic microcentrifuge tubes and was difficult to wash away with PBS. Such a property of adherence of efavirenz may have affected measurements of intracellular quantities in the previous protocols.

First, the efflux rate of efavirenz from cells was studied. PBMCs isolated from a healthy donor by use of Ficoll-Paque Plus solution (GE Healthcare, Piscataway, NJ) were incubated in 1 μM efavirenz in serum-free VP-SFM medium (Invitrogen, Carlsbad, CA) at 37°C for 1 h and then transferred to drug-free medium and incubated at either 4°C or 37°C for up to 20 min. Three aliquots of 10^6 cells were taken at each time point, and the efavirenz contained in each aliquot was extracted with 80% methanol and quantified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) with a high-performance LC system (Agilent 1100 Series; Agilent, Palo Alto, CA) and a tandem mass spectrometer (API QStar Pulsar *I*; Applied Biosystems, Foster City, CA) using electrospray ionization in which an *m/z* transition of 333 to 272 atomic mass units for $(M+NH_4)^+$ precursor ions of efavirenz was used. As shown in Fig. 1, the intracellular efavirenz concentration declined within 5 min at either 37°C or 4°C to a level near the background level. This result poses another question about the previous isolation procedures used for PBMCs, because they were based on the assumption that drug efflux produced during washing procedure is suppressed by the use of ice-cold PBS. This result, taken together with those demonstrating the highly lipophilic property of efavirenz, suggests that intracellular efavirenz is eluted from the cells and adsorbed to the surface of plastic vessels during cell washing. To examine this hypothesis we compared quantifications of levels of efavirenz in PBMCs by two different isolation protocols: one used the same tube during washing, and the other used new tubes in each washing (Table 1). Transfer of cell suspension to a new tube each time reduced the efavirenz quantity remarkably compared with the use of the same tube throughout washing. These results suggest that the drug exudes from cells during cell washing and that the drug present in residual plasma was not washed away in studies employing the previously used procedures.

Thus, we decided to develop a new method for isolation of PBMCs (Fig. 2). The method consists of three steps: banding of PBMCs at the bottom of the plasma layer but not in the Ficoll-Paque solution by a relatively short centrifugation time; washing of PBMCs with concurrently prepared plasma of the same patient; and separation of PBMCs from the plasma by centrifugation through silicone oil (supplemented with 8% *n*-hexadecane) in a "double tube." By this method, efavirenz in PBMCs are kept equilibrated with the efavirenz in plasma until

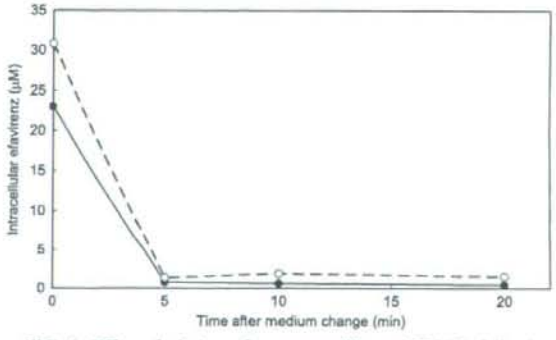

FIG. 1. Efflux of efavirenz from preequilibrated PBMCs following incubation in drug-free medium at 37°C (closed circles) and 4°C (open circles).

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TABLE 1. Quantification of efavirenz in PBMCs isolated by the present method and a previously reported method[a]

Source	Intracellular efavirenz concn[b] (μM)		
	Previous method with one tube[c]	Previous method with new tubes[d]	Present method[e]
Spiked donor blood[f]	110 ± 19	4.6 ± 1.7	97 ± 17
Patient 1	Not assayed	3.8 ± 1.3	63 ± 24
Patient 2	Not assayed	1.1 ± 0.3	71 ± 11

[a] The method reported by Almond et al. (1) was used for isolation of PBMCs for comparison with the present method.
[b] Efavirenz was quantified by LC-MS/MS. Intracellular concentrations were calculated, taking 0.25 pl as the volume of a single cell (5). Data are expressed as means ± standard deviations (n = 3).
[c] Washing of PBMCs in ice-cold PBS was carried out three times in the same microcentrifuge tube.
[d] Suspension of PBMCs in ice-cold PBS was transferred into a new microcentrifuge tube for centrifugation before each of three washings.
[e] Intracellular efavirenz concentrations were determined as described in the legend to Fig. 2.
[f] HIV-uninfected donor blood was spiked with efavirenz at 10 μM.

PBMCs are separated from the plasma. The use of a "double tube" in centrifugation prevents plasma from contaminating the surface of an outer microcentrifuge tube and thus from getting into the extract of PBMCs.

By using this method, we studied the dependence of intracellular efavirenz levels on the concentration of plasma in medium containing efavirenz at 10 μM. Efavirenz not bound by protein was obtained by use of a Centrifree micropartition device (Centrifree, Billerica, MA). The intracellular efavirenz concentration decreased with an increase in the plasma concentration (Fig. 3A) and was proportional to the unbound efavirenz concentration in medium, with a mean intracellular accumulation ratio of 970 (Fig. 3B). These results show that the major determinant of intercellular efavirenz levels is extracellular unbound levels but not total extracellular levels, which agrees with the pharmacological idea that only unbound drug is able to enter the cell.

Next, the relationships between total plasma, unbound plasma, and intracellular efavirenz concentrations were studied using samples from 13 patients receiving antiretroviral agents containing efavirenz in Keio University Hospital (Tokyo, Japan). The patients provided written informed consent for the study, which was approved by the local ethics committee. The median duration of treatment was 15 months, with a range from 1 to 65 months. Concurrent drugs used for treatment were zidovudine and lamivudine (3TC) for four patients, stavudine and 3TC for five, tenofovir disoproxil fumarate and emtricitabine for three, and tenofovir disoproxil fumarate and 3TC for one. All patients were Japanese males and exhibited no biochemical evidence of impairment of the liver or kidney. Although there were significant associations between total and unbound plasma levels (Spearman's rank correlation coefficient [rs] = 0.66; P = 0.021) (Fig. 4A) and between total plasma and intracellular levels (rs = 0.66; P = 0.021) (Fig. 4B), a stronger correlation was observed between unbound plasma and intracellular levels (rs = 0.76; P = 0.0082) (Fig. 4C), with a mean accumulation ratio of 1,190.

The results presented above are contrasted with the results

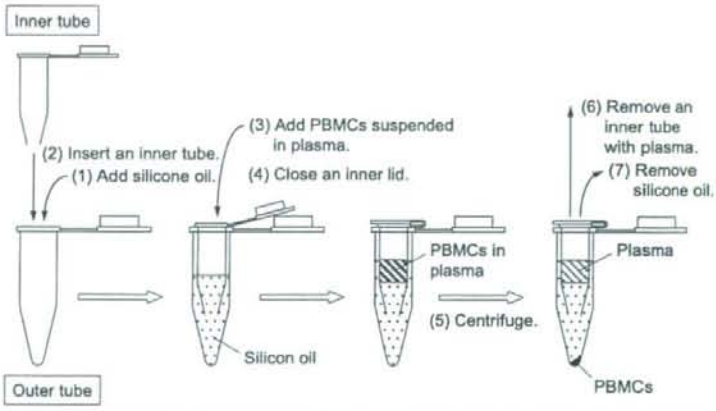

FIG. 2. Preparation of PBMCs for intracellular efavirenz quantification. Anticoagulated blood (1 ml) was layered on 800 μl of a Ficoll-Paque Plus solution (GE Healthcare, Piscataway, NJ) and centrifuged at 180 × g for 10 min. After centrifugation, PBMCs which formed a layer at the boundary between plasma and Ficoll-Paque Plus solution were collected and centrifuged at 320 × g for 2 min. Precipitated PBMCs were washed in 100 μl of plasma prepared from the same blood and centrifuged at 500 × g for 2 min. PBMCs were separated from plasma as follows: (i) a mixture of silicone oil (catalog no. SH-550; Nacalai, Kyoto, Japan) and n-hexadecane (Nakalai) (800 μl; 92:8 [vol/vol]) was added into an outer tube (1.5-ml microcentrifuge tube); (ii) an inner tube (made from a 0.5-ml microcentrifuge tube) with a hole at the bottom was sunk in silicone oil in the outer tube; (iii) PBMCs suspended in 100 μl of plasma were layered on silicone oil in the inner tube; (iv) the lid of the inner tube was pressed down; (v) an assembled "double tube" was centrifuged at 16,000 × g for 1 min, thereby precipitating PBMCs quickly to the bottom of the outer tube; (vi) the inner tube containing plasma and silicone oil was removed, with the lid kept closed to prevent plasma falling into the outer tube; and (vii) silicone oil was removed while leaving pelleted PBMCs in place and then the remaining silicone oil on the tube surface was removed after a brief centrifugation. Pelleted PBMCs were suspended in 30 μl of water, and immediately 10 μl of the suspension was used for determination of cell numbers with a cell counter (Celltac; Nihon Kohden, Tokyo, Japan); note that suspending cells in PBS is not recommended, because the presence of phosphate ions in samples affects ionization of molecules in mass spectrometry and sometimes causes severe troubles to the machine. To the remaining cell suspension, 80 μl of methanol was added, and efavirenz was quantified by LC-MS/MS.

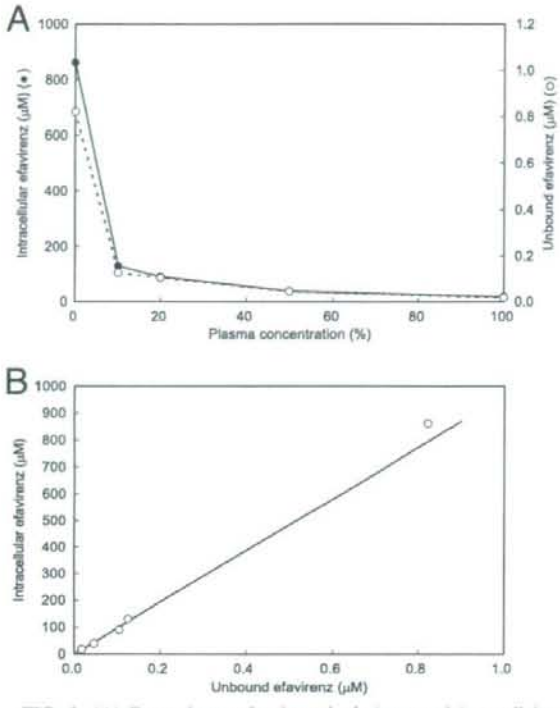

FIG. 3. (A) Dependence of unbound efavirenz and intracellular efavirenz levels on plasma content (expressed in percentages) in medium containing efavirenz at 10 μM. (B) Relationship between unbound and intracellular efavirenz concentrations as deduced from the data presented in panel A.

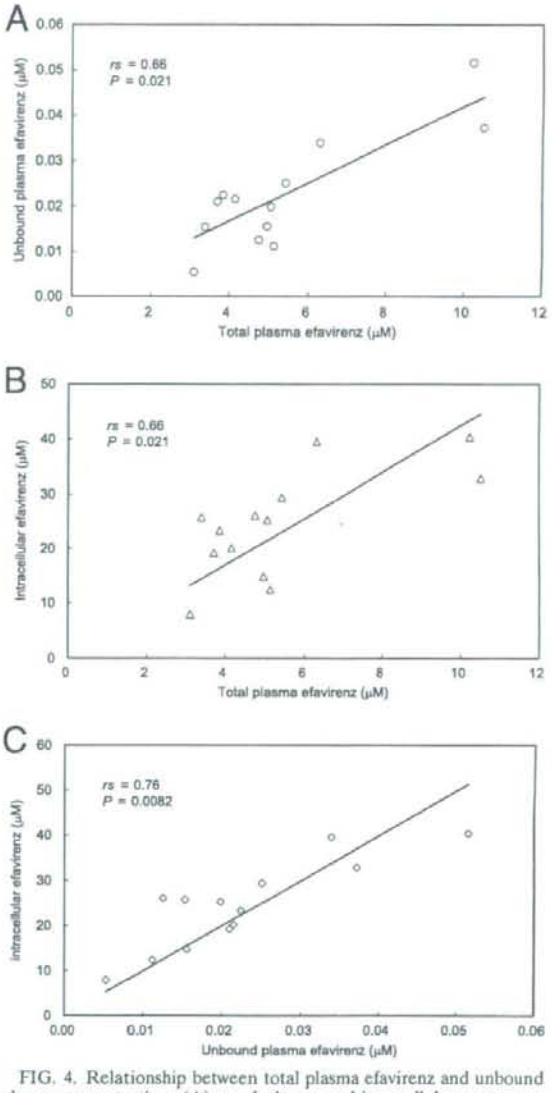

FIG. 4. Relationship between total plasma efavirenz and unbound plasma concentrations (A), total plasma and intracellular concentrations (B), and unbound plasma and intracellular concentrations (C) in samples from 13 patients receiving antiretroviral therapy that included efavirenz treatment. Data were analyzed by Spearman rank correlation.

of previous studies (1, 10) and demonstrate that intracellular efavirenz levels are not correlated with unbound plasma levels but are significantly correlated with total plasma levels. They indicate that total plasma efavirenz concentrations may be good surrogate markers for intracellular concentrations and that binding of efavirenz to plasma protein may be linked to its binding to unknown materials in cells. It should be noted that these studies used similar methods for isolation of PBMCs in which PBMC extract is, as argued above, apt to be contaminated with plasma efavirenz. If such contamination occurred in their studies, it would be inevitable that a stronger correlation would be observed between total plasma and intracellular efavirenz levels.

Efavirenz was highly accumulated in resting PBMCs. This may have been due to the binding to cellular protein or membrane because of its high lipophilicity ($\log_P$ = 5.4). Efavirenz bound to cellular components is unlikely to play a direct role in inhibition of reverse transcriptase activity. To study further the biological significance of intracellular drugs, new technologies for quantifying intracellular unbound drug and for identifying intracellular drug localization are required.

Most protease inhibitors also have the properties of rapid excretion from cells and high lipophilicity (4, 8). It may be necessary to examine whether the procedures for intracellular quantification of these agents (6, 7) suffer from the possible artifacts suggested for efavirenz in this study.

In conclusion, we have developed an intracellular efavirenz quantification method which overcomes spontaneous drug elution from cells and contamination with drug-containing plasma. The present method may be a useful tool for elucidating the therapeutic relevance of intracellular levels of efavirenz.

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Buoyant density and sedimentation dynamics of HIV-1 in two density-gradient media for semen processing

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Objective: To compare buoyant density and sedimentation kinetics of human immunodeficiency virus 1 (HIV-1) in two sperm-washing media, Percoll and Pureception.
Design: Laboratory study.
Setting: University hospital.
Patient(s): None.
Intervention(s): Buoyant density and sedimentation kinetics of HIV-1 particles (MOLT-4/LAI strain) were measured in Percoll and Pureception using isopyknic ultracentrifugation and continuous-density-gradient centrifugation.
Main Outcome Measure(s): The HIV-1 particles were detected and semiquantified using a reverse transcription polymerase chain reaction (RT-PCR) for HIV-1 RNA.
Result(s): Calculated buoyant density of HIV-1 was approximately 1.042 in both media in isopyknic centrifugation. However, most HIV-1 particles were found in fractions with specific gravity less than 1.04 in both media, even after 40 minutes of density-gradient centrifugation at 1,600 *g*. Small viral accumulations were observed at the bottom of the tube in Pureception density gradients.
Conclusion(s): Although we found very high efficiency of HIV-1 removal using density-gradient centrifugation, a minute quantity of virus was found at the bottom of the gradient tube when Pureception was used as the medium. (Fertil Steril® 2008;90:1983–7. ©2008 by American Society for Reproductive Medicine.)

Key Words: Sperm, Percoll, Pureception, density-gradient centrifugation, HIV-1, isopyknic centrifugation

Transmission of human immunodeficiency virus type 1 (HIV-1) through semen is widely recognized. Many studies have demonstrated that HIV-1 exists both as free virus in the seminal plasma and as cell-associated virus in semen (1–8). Sperm preparation techniques originally developed to separate the fraction containing highly motile spermatozoa from that of seminal plasma and nonmotile cells have been reported to reduce HIV-1 RNA and proviral DNA to undetectable concentrations (9, 10).

Increasingly, serodiscordant couples with an HIV-1–positive husband and an HIV-1–negative wife are requesting insemination techniques for avoiding risk of transmission to the female partner and to offspring (11). Intrauterine insemination (IUI), in vitro fertilization (IVF), and intracytoplasmic sperm injection (ICSI) after sperm preparation by techniques commonly referred to as "sperm washing" have been suggested as ways to reduce likelihood of transmission of HIV-1. Numerous pregnancy successes without HIV-1 seroconversions have been reported (12–14).

Sperm washing as the first step of the virus elimination procedure has used density-gradient centrifugation. Because HIV-1 infection is believed to worsen the condition of sperm, men with chronic HIV-1 infection often have abnormal semen profiles (15). An optimal washing method would maximize recovery of spermatozoa without decreasing viral removal efficacy. To the best of our knowledge, however, precise reliable data have not been obtained concerning buoyant density or sedimentation kinetics of HIV-1 in density-gradient media such as polyvinylpyrrolidone (PVP)-coated colloid-silica gel (Percoll) or silane-coated colloid-silica gel (Pureception). The latter medium has been used increasingly in place of Percoll.

In the present study, we determined the buoyant density of free HIV-1 in each of two sperm-washing media, Percoll and Pureception, using a highly sensitive nested competitive reverse transcription polymerase chain reaction (RT-PCR) technique that we established previously (10). Sedimentation dynamics in continuous density gradients using these two media also were investigated.

MATERIALS AND METHODS

Reagents

Each concentration of Pureception solution was prepared by diluting 90% Pureception with sperm-washing medium (both from Sage In Vitro Fertilization, Pasadena, CA). Each concentration of Percoll solution was prepared by diluting 80% Percoll according to the method of Kaneko et al. (16), using 10 mmol/L HEPES-buffered Hanks solution

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(pH 7.4) including 4.0 mg/mL human serum albumin and antibiotics (0.14 mg/mL latamoxef sodium and 0.11 mg/mL ampicillin).

Viral RNA Quantification

A concentrated suspension of wild-type HIV-1 LAI strain produced from chronically infected MOLT-4 cells was used as the source of virus (10). As a quantification method for HIV-1, we used a nested competitive RT-PCR technique that we developed previously (10). Briefly, the internal competitor DNA consisted of an HIV-1 *env* sequence (nucleotide positions 6201 to 8805 according to NL4–3) (17) with deletion of positions 7119 to 7241 and addition of a T7 promoter (TAA TAC GAC TCA CTA TAG GGA GA) at the 5′ terminus. Internal competitor RNA was synthesized from the competitor DNA with T7 RNA polymerase. The DNA and RNA were quantified by spectrophotometry and end point dilution followed by Poisson analysis of positive scores from nested PCR, as described previously. Using this method, viral load has been confirmed to be maintained consistently at approximately 10 copies/20 μL.

Determination of Buoyant Density of HIV-1 by Isopyknic Focusing

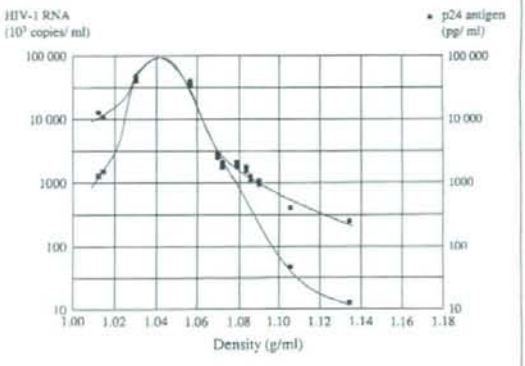
The HIV-1 LAI strain (0.2 mL) was mixed with 2.5 mL 65% Percoll or 2.5 mL 50% Pureception (final viral concentration 1.15×10^6/mL). After centrifugation at 16,400 *g* (Percoll) or 11,400 *g* (Pureception) for 20 minutes, aliquots of the postcentrifugation suspension (each approximately 0.25 mL) were fractionated beginning at the bottom of the tube. Mean specific gravity of each fraction was determined by weighing 100 μL of each solution using a measuring micropipette. Viral concentration was determined by nested competitive RT-PCR, and p24 antigen was quantified with MiniVidas (Biomerieux, Marcy l'Etoile, France). Buoyant density of HIV-1 particles was determined from the solvent density corresponding to a peak of viral distribution.

Preparation of Continuous Density Gradients with a Gradient Pump and Evaluation of Sedimentation Dynamics

Two pairs of solutions as described subsequently were mixed in a pump for column preparation (Bio-Rad, Philadelphia, PA) to prepare 3-mL continuous density gradients: either a mixture of 80% Percoll and Hanks solution or a mixture of 90% Pureception and sperm-washing medium. A 0.2-mL aliquot of the concentrated HIV-1 suspension was layered on top of the upper layer of the prepared density gradient. After centrifugation at 1,600 *g* for 5, 10, 20, and 40 minutes, aliquots of the postcentrifugation suspension (approximately 0.25 mL) were fractionated beginning at the bottom of the tube. Mean specific gravity was determined, and viral load in each fraction was quantified using the nested competitive RT-PCR technique.

FIGURE 1

Viral RNA load in each density fraction of Percoll. Fractions were collected from the bottom and analyzed for density, human immunodeficiency virus 1 (HIV-1) RNA (*squares*), and p24 (*circles*). The HIV-1 RNA and p24 amounts were plotted against the density of each fraction, using a semilogarithmic scale. Both HIV-1 RNA and p24 showed a single peak at the position representing a density of 1.042.



Kuji. Sedimentation kinetics of HIV-1. Fertil Steril 2008.

RESULTS

Buoyant Density in Percoll and Pureception

Viral RNA amounts, p24 antigen amounts, and medium density were determined in each of 12 fractions of a centrifuged mixture of HIV-1 LAI strain and 65% Percoll. Viral RNA and p24 antigen amounts were plotted against measured medium density using a semilogarithmic scale (Fig. 1). Assuming viral distribution curves to be normally distributed, distribution peaks of HIV-1 RNA and p24 antigen were calculated to exist at approximately 1.042 g/mL. Accordingly, the buoyant density of HIV-1 in Percoll was estimated to be 1.042 g/cm^3. Data for Pureception are presented in Figure 2. The buoyant density of HIV-1 in Pureception was estimated to be 1.042 g/cm^3, the same value as with Percoll. Viral distribution had a single peak in Percoll but showed two peaks in Pureception, with a small amount of virus in the higher-density fraction (bottom of the tube) in addition to the main peak.

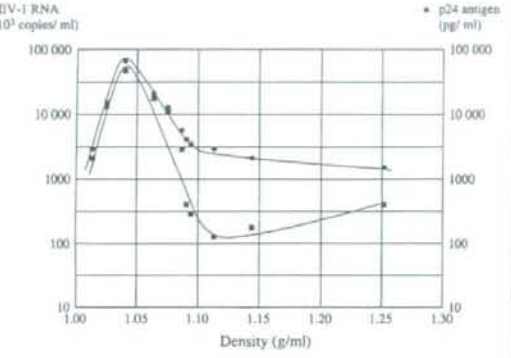
Sedimentation Dynamics in Percoll and Pureception

High reproducibility was observed in the two continuous density gradients ranging from 0% to 90% Pureception and 0% to 80% Percoll. Linearity was maintained even after centrifugation at 1,600 *g* for 40 minutes (data not shown).

Using the continuous density gradients described, sedimentation dynamics of HIV-1 layered on the top of the upper layer of each gradient were investigated. Most HIV-1 particles were found in fractions with specific gravity less than 1.04, even after 40 minutes of centrifugation, in both continuous density gradient media (Fig. 3). In Pureception,

FIGURE 2

Viral RNA load in each density fraction of Pureception. Fractions were collected from the bottom and analyzed for density, human immunodeficiency virus 1 (HIV-1) RNA (*squares*), and p24 (*circles*). The HIV-1 RNA and p24 amounts were plotted against the density of each fraction, using a semilogarithmic scale. Amounts of p24 showed a single peak as for Percoll. The HIV-1 RNA amounts, however, showed two peaks: a small amount of virus at the higher-density position (the bottom of the centrifugation tube) in addition to the main peak.

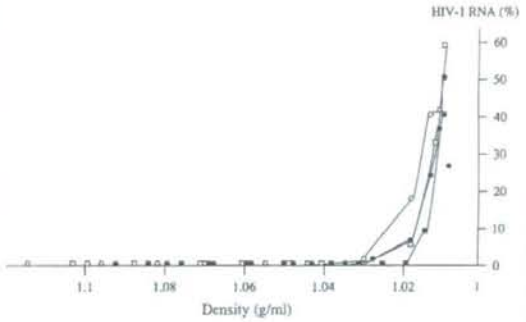


Kuji. Sedimentation kinetics of HIV-1. Fertil Steril 2008.

however, small viral accumulations were observed at the bottom of the tube (data not shown), a finding absent with Percoll.

FIGURE 3

The human immunodeficiency virus 1 (HIV-1) viral load after centrifugation in Percoll and in Pureception (1,600 *g*). Concentrated HIV-1 suspension was layered on top of either Percoll or Pureception density gradient and centrifuged at 1,600 *g* for 5, 10, 20, or 40 minutes. Beginning at the bottom of the tube, each 0.25-mL aliquot was removed as a fraction and then analyzed. Viral load after centrifugation for 10 minutes (*sold squares:* Percoll; *open squares:* Pureception), and 40 minutes (*solid circles:* Percoll; *open circles:* Pureception) are shown. Most HIV-1 particles were present in fractions with specific gravity near or below 1.04, even after 40 minutes of centrifugation in continuous density gradients using both Percoll and Pureception. With Pureception, however, small viral accumulations were observed at the bottom of the tube (data not shown), a finding absent with Percoll.



Kuji. Sedimentation kinetics of HIV-1. Fertil Steril 2008.

DISCUSSION

When the male partner is HIV-1 positive, a female partner who is HIV-1 negative is estimated to incur a 0.1% to 0.2% risk of acquiring HIV-1 per act of unprotected intercourse (18). Thus, attempting to conceive naturally carries a serious risk to the uninfected female partner and to the child (19). Density-gradient centrifugation techniques can effectively eliminate HIV-1 from semen to reduce the risk of HIV-1 transmission in HIV-1–discordant couples wishing to have children (9, 10). Although Baccetti et al. (3) detected HIV-1 particles and HIV-1 nucleic acid in ejaculated washed sperm preparations from HIV-1–seropositive patients, other investigators noted total absence of HIV-1 particles and nucleic acid in washed sperm (1, 7, 20). Thus, separation of seminal fluid and cellular elements from sperm by washing techniques reduces the viral load of semen detected by PCR and RT-PCR. Consequently, several groups have achieved pregnancy successes without HIV-1 seroconversions by using sperm-washing methods (12, 13).

Separation of spermatozoa using density-gradient centrifugation is possible in part because spermatozoa are motile, unlike other particulates present; additionally, sedimentation velocities differ between spermatozoa, seminal plasma, lymphocytes, and free virus. Highly motile spermatozoa migrate more quickly than nonmotile constituents during density-gradient centrifugation, because centrifugal force orients them with head downward and tail upward (21). Because motility thus contributes importantly to separation, efficiency ordinarily is enhanced for spermatozoa owing to their motility. In contrast, for spermatozoa with impaired motility, especially those from HIV-1–positive men with infertility from factors such as oligozoospermia or asthenozoospermia, buoyant density as well as sedimentation velocity of the virus in sperm-washing media become the most important factors for separating virus from sperm. With poorly motile spermatozoa, density-gradient viral separation is likely to fail unless centrifugation-related factors are carefully considered.

In the present study, we investigated sedimentation dynamics of HIV-1 in two extensively used sperm-washing media, Percoll and Pureception. Percoll, a colloidal suspension of PVP-coated silica particles, has been used widely for sperm processing in assisted reproductive technology, offering

simplicity, rapidity, and excellent yields. In 1996, however, Percoll was withdrawn from clinical use in humans because of concern over possible endotoxin contamination. Pureception, a colloidal suspension of silane-coated silica particles developed as a substitute, has been shown to be effective in recovering motile sperm by several studies comparing it with Percoll, and it is now used worldwide (22–26).

The buoyant density for HIV-1 in a linear sucrose gradient was reported to be 1.16 g/mL (27), which differs from the buoyant density of 1.04 g/mL obtained in the present experiment with both sperm-washing density gradient media, Percoll and Pureception. In isopyknic focusing, buoyant densities of particles can vary considerably according to the composition of the density gradient media used, reflecting differences in osmolarity of the medium (28). We therefore took care to use the same density gradient media actually used for sperm washing in determining the buoyant density of HIV-1. This value proved to be equal between the two sperm washing media, Percoll and Pureception, probably because both consist mainly of silica gel. Their osmotic pressures were kept equal in view of their having a common use, processing of spermatozoa.

We also examined the sedimentation velocity of HIV-1 in continuous density gradients made of Percoll and Pureception. These continuous-gradient columns, prepared with a gradient pump, demonstrated high reproducibility and post-centrifugation stability. In clinical settings, use of discontinuous density gradients is quite common, whereas continuous density gradients are rarely used, because highly specialized equipment and personnel are required to prepare reproducible gradients. However, with discontinuous density gradients, virus may be trapped at the interface between densities and the sedimentation rate of the virus also may change as a result of viral aggregation–especially when high concentrations of virus are applied, as in the present study. For that reason, we used continuous density gradients for determination of the distribution of viral particles among the sedimentation fractions. High concentrations of HIV-1 were layered on the upper surface of the continuous density gradient before centrifugation. Even after centrifugation at 1600 *g* for 40 minutes, almost all HIV-1 virus particles remained in the superficial fractions where density was near or less than 1.042, the determined buoyant density of HIV-1. This confirmed that the sedimentation velocity of HIV-1 was very low in continuous density gradients made up from either Percoll or Pureception.

Interestingly, minute amounts of virus were found to be clustered at the bottom of the Pureception gradient tube but not the Percoll gradient tube. The reason for this difference remains unknown, but one possible explanation might involve a difference in silica particle size between Percoll and Pureception. The average diameter of Percoll particles is about 17.2 nm, and the size of the particles in Pureception before silane coating is about 15 nm; the density-gradient particles in Percoll would appear to be slightly larger than those in Pureception. Another possible explanation might involve differences in the substances coating the silica particles. Silane (in Pureception) might exert slightly less friction against various particles than PVP (in Percoll). In fact, the recovery rate of spermatozoa has been reported to be greater using Pureception than using Percoll (22). The decreased friction in Pureception might permit small HIV-1 aggregates to form.

Effectiveness of clinical management of HIV-1–serodiscordant couples using sperm washing followed by a swim-up technique is presently under investigation. After carrying out artificial insemination in more than 300 cases, Semprini et al. (12) reported that transmission of infection was prevented successfully in all subjects. In contrast, another study found transmission of infection in some cases where the semen was subjected only to a washing procedure (29). Although several problems remain to be solved, the American Society for Reproductive Medicine recommended in 2004 that procedures for HIV-1–discordant couples wanting a child can be performed, but only at institutions able to provide the most effective methods of sperm preparation as well as the rigorous testing and treatment necessary to minimize the chance of HIV-1 transmission to partner and offspring (30).

In conclusion, we investigated the buoyant density of HIV-1 and dynamic changes in the sedimentation pattern of free HIV-1 in both Percoll and Pureception media for "sperm washing." We found very high efficiency of free virus removal using either medium for density-gradient centrifugation. However, a minute quantity of virus was found at the bottom of the gradient tube in addition to the virus retained in overlying fractions when Pureception was used as the medium. Accordingly, washing procedures used clinically must be assessed individually in terms of specific type of density gradient medium.

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Increasing genetic diversity of hepatitis C virus in haemophiliacs with human immunodeficiency virus coinfection

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Patients with inherited bleeding disorders who received clotting factor concentrates before 1987 have high rates of hepatitis C virus (HCV) or HCV/human immunodeficiency virus (HIV) infection. To determine whether the persistent nature of HIV affects the genetic diversity of HCV by less selective pressure through the immunosuppression of HIV/HCV-coinfected patients, both the change of genetic diversity and selective pressure were examined in the HCV envelope genes (E1 and E2) of 325 genotype 1a subclones from eight HIV-positive and five HIV-negative patients with two time points (more than 6 years apart). To infer the genetic diversity of HCV in each patient, we used two approaches. One method was to estimate the difference of total evolutionary distances in the phylogenetic tree between the two time points, and another was to estimate the changes of genetic diversity along the time based on the coalescence theory. The two results indicate that the HIV-positive group has significantly more diverse population structure than the HIV-negative group. A comparative analysis of the synonymous and non-synonymous substitutions found that the HIV-positive group was subject to less selective pressure than the HIV-negative group. In conclusion, HIV-positive patients would have a more diversified HCV population than HIV-negative patients due to less selective pressure from the immune system.

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INTRODUCTION

Increased rates of progression to end-stage liver disease, mortality and reduced treatment response rates have been well documented in haemophiliac and other groups of chronic hepatitis C virus (HCV) carriers with human immunodeficiency virus (HIV) coinfection (Bica *et al.*, 2001; Goedert *et al.*, 2002; Braitstein *et al.*, 2004). Although the mechanism of liver disease progression in HIV-infected patients remains unclear, one of the important roles is assigned to immunosuppression (Goedert *et al.*, 2002).

The estimated HCV virion half-life time was, on average, 2.7 h with pre-treatment production and clearance of 10^{12} virions per day (Neumann *et al.*, 1998). Such a high rate of HCV replication, combined with lack of an error correction mechanism, results in the development of genetically diverse clones in a patient. The genetic diversity of HCV has provided critical insights into short-term outcomes, including early spontaneous viral clearance (Farci *et al.*, 2000), interferon-associated viral clearance (Farci *et al.*, 2002; Pawlotsky *et al.*, 1999) and HCV emergence following liver transplantation (Lyra *et al.*, 2002). To infer the genetic diversity of HCV in a patient, we applied two approaches. One method simply assumed that the genetic diversity of HCV is of different divergence of synonymous distance between two time points. The other method applied to coalescent analysis of genetic diversity along the time, assuming that the genetic diversity of HCV represents a heterogeneous viral population in a given carrier. To evaluate the influence on the HCV evolution exerted by the immunosuppression during persistent HIV coinfection, we examined a cohort of HCV carriers, comparing the HIV-positive and -negative groups.

Determination of the antigen-recognition regions associated with HCV-specific immune positive selection is important for understanding selective pressures underlying the evolution of HCV as well as putative therapeutic targets. In this study, we evaluated the genetic diversity of HCV and determined genomic regions associated with positive selection by comparative analysis of selective forces between HIV-positive and -negative groups in a cohort of haemophilia patients followed for more than 6 years.

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this study are AB245555–AB245873.