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BRIEF COMMUNICATION

Preferential expression and immunogenicity of HIV-1 Tat fusion protein expressed in tomato plant

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Abstract HIV-1 Tat plays a major role in viral replication and is essential for AIDS development making it an ideal vaccine target providing that both humoral and cellular immune responses are induced. Plant-based antigen production, due to its cheaper cost, appears ideal for vaccine production. In this study, we created a plant-optimized *tat* and mutant (Cys30Ala/Lys41Ala) *tat* (*mtat*) gene and ligated each into a pBI121 expression vector with a stop codon and a *gusA* gene positioned immediately downstream. The vector construct was bombarded into tomato leaf calli and allowed to develop. We thus generated recombinant tomato plants preferentially expressing a Tat-GUS fusion protein over a Tat-only protein. In addition, plants bombarded with either *tat* or *mtat* genes showed no phenotypic difference and produced 2–4 μg Tat-GUS fusion protein per milligram soluble plant protein. Furthermore, tomato extracts intradermally inoculated into mice were found to induce a humoral and, most importantly, cellular immunity.

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Keywords AIDS · Antibody response · Cellular immune response · HIV-1 · Tat · Transgenic tomato

Introduction

HIV-1 has already claimed millions of victims worldwide and despite billions of dollars spent on HIV-1/AIDS research annually (Walker and Burton 2008; Watkins et al. 2008), no promising candidate HIV-1 vaccine has been made to date due to: (a) specific viral characteristics including extreme genetic variability among various isolates collected worldwide and even within the infected individuals; (b) a high mutation rate allowing rapid escape of variants from immune responses; and (c) biological properties of HIV-1 regulatory proteins, such as Nef and Tat, which avoid immune responses (Walker and Burton 2008; Watkins et al. 2008; WHO 2008; Potts et al. 2008). As widely believed, these characteristics pose a major obstacle towards controlling AIDS (Gaschen et al. 2002; Moore et al. 2008).

An ideal strategy against HIV-1 is one that stimulates passive protection or neutralizing immunity by

producing both antibodies and cytotoxic T lymphocytes or CTLs (Walker and Burton 2008; Watkins et al. 2008; Addo et al. 2001). Earlier works have shown that CTLs can control HIV-1 replication in the absence of antibodies (Borrow et al. 1994) prompting several attempts to stimulate anti-viral CTL responses using a combination of varying HIV-1 proteins and their epitopes (Betts et al. 2005; Matano et al. 2004; Mwau et al. 2004). The Tat protein has been one of the well studied HIV-1 proteins (Barboric and Peterlin 2005; Emerman and Malim 1998; Goldstein et al. 2001; Okamoto and Wong-Staal 1986; Ramirez et al. 2007). It is a small regulatory protein composed of either 86 or 101 amino acid residues (14 or 18 kDa, respectively) encoded by two exons (Okamoto 1995). Among the HIV-1 proteins already studied, Tat shows great potential for CTL induction covering a wide variety of HIV-1 clones besides from little variability among distinct viral subtypes and is highly conserved in both inter- and intra-patient variants (Addo et al. 2001; Goldstein et al. 2001).

Over 4 million people become infected with HIV-1 each year (WHO 2008; Fox 2007) in third-world countries in particular (Flexner 2008). Cheap and affordable production of pharmaceutical products for third-world consumption has prompted the development of plant-made pharmaceuticals for often neglected diseases (Zahn et al. 2008), including HIV-1 (Ramirez et al. 2007; Flexner 2008; Shchelkunov et al. 2006; Webster et al. 2005). Previous attempts to utilize the tomato plant for HIV-1 Tat vaccine development in the form of an edible-vaccine was only successful in inducing antibodies or humoral immune response (Ramirez et al. 2007; Shchelkunov et al. 2006). At present, no report has been made with regards to induction of CTLs or cellular immune responses using Tat protein (Addo et al. 2001), more so, using a plant-expressed Tat protein.

In this study, we demonstrate the evidence of preferential expression of a Tat-GUS fusion protein over the Tat-only protein in tomato plant and is expressed much higher than previously reported (Ramirez et al. 2007). In addition, we were able to induce both humoral immune response and, surprisingly, cellular immune response using Balb/c mice when tomato extracts were intradermally introduced. To our knowledge, this is the first report of cellular immune induction using Tat expressed in a plant system.

Materials and methods

Vector construction and tomato transformation

The *tat* gene from the HXB2 strain of HIV-1 and *mtat* were synthesized following a specific codon-usage table based on tomato was used (www.kazusa.or.jp/codon/cgi-bin/showcodon.cgi?species=4081). The plant-optimized M2 epitope directly fused to either *tat* or inactive *mtat* (Imai et al. 2005) genes with a stop codon, were individually ligated into a pBI121 expression vector (Clontech) upstream of a *gusA* gene (Fig. 1). Transformation was performed using a particle gun

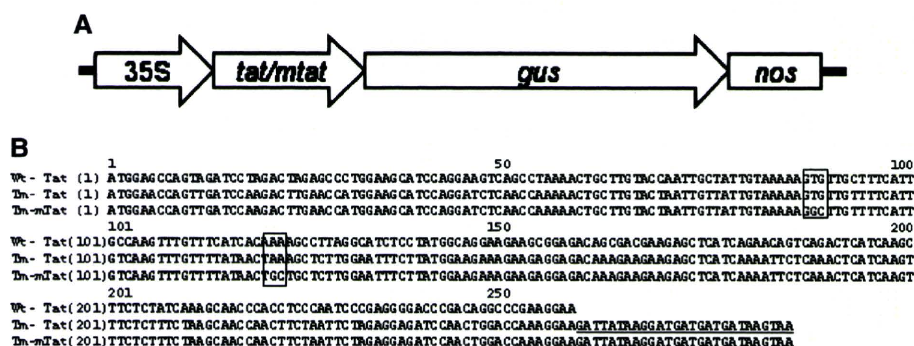

Fig. 1 Construction of the pBI121 plant expression vector containing either *tat* or *mtat* indirectly fused to *gusA* gene. **a** Expression was driven by 35S CaMV promoter and terminated with NOS termination signal located downstream of *gusA* gene. The inserted *tat/mtat* gene is located upstream of the *gusA* gene containing the termination codon (TAA) in between. **b** Codon-optimized *tat* and *mtat* were synthesized following the codon usage of tomato. The *boxed regions* represent point mutations at Cys30Ala and Lys41Ala found in *mtat* [23]. The *underlined segment* represents M2 epitope added to serve as an expression tag. Wt-Tat represents the Tat sequence from the HXB2 strain. Tm-Tat and Tm-mTat represents codon-optimized tomato Tat and mTat, respectively

(Tanaka Co., Ltd, Tokyo, Japan) and the tomato var. *Improved Pope* as previously published (Bhatia and Ashwath 2004; Sheeja et al. 2004). Briefly, the sterilized seeds were grown in MS medium and allowed to grow for 7–10 days. Callus induction of the explant material was performed in a MS medium containing 1.0 ppm zeatin for 7 days. Bombardment was carried out in tomato calli grown in MS medium containing 0.5 ppm zeatin, 1.0 ppm indole-butyric acid and 1.0 ppm giberillic acid. All reagents used for tomato transformation were purchased from Sigma.

Reverse transcription-PCR

Triplicates of bombarded tomato calli, regenerated leaf and shoot tissues were freshly obtained for mRNA extraction. The MicroFastTrack™ 2.0 mRNA Isolation Kit (Invitrogen) was used to isolate mRNA according to manufacturer's instructions. The Cloned AMV First-Strand cDNA Synthesis Kit (Invitrogen) was used to synthesize cDNA according to manufacturer's instructions. The forward primer, TAT-F (5′-ATG GAA CCA GTT GAT CC-3′), used was based on the tomato codon-based HIV-1 *tat*, whereas, the reverse primer, GUS-R (5′-CGG TAT AAA GAC TTC GCG CTG-3′) was based on *gusA*. Both primers were synthesized by Invitrogen. The TOUCHDOWN PCR condition was performed using the TaKaRa *Taq*™ Hot Start Version (Takara Bio Inc., Japan) with an initial denaturation temperature of 95°C for 5 min proceeded by 5 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. This was followed by another 5 cycles of 94°C for 1 min, 53°C for 1 min and 72°C for 2 min. The last set of cycles consists of 25 cycles at 94°C for 1 min, 50°C for 1 min and 72°C for 2 min. A final 10 min extension at 72°C was also performed. Reverse transcription-PCR (RT-PCR) products were resolved on 1% agarose gel.

Protein extraction

Protein extracts were obtained from 2-weeks old transgenic tomato plants. Protein extraction was done on all transgenic samples using the P-PER Plant Protein Extraction Kit (Thermo Scientific) according to manufacturer's recommendation. Tomato protein extracts acquired ($\sim$100 μl) were divided for use in Western blot assay and Immunogenicity testing.

Western blot assay

Western blot using both antibodies against Tat and M2 (Sigma) was performed and amount of Tat expressed in bombarded tomato plants were estimated using the Bio-Dot Mircofiltration Apparatus (BIO-RAD) as previously published (Ota et al. 2005). The Tat protein standard used was a recombinant Tat (ImmunoDiagnostics, Inc.) with various dilutions.

Immunogenicity testing

Balb/c mice were intradermally immunized with the recombinant tomato protein extracts mixed in an incomplete Freund's adjuvant (IFA). The peptides used in this study were the Tat CTL epitope (Morris et al. 2001) and B cell epitope (Goldstein et al. 2001). Tat-specific antibody responses were measured by ELISA. Briefly, synthetic peptides for Tat and mutant Tat (mTat) diluted in PBS were coated in multiwell plates overnight at 4°C followed by 30 min of blocking with non-fat milk. Test samples were then added and incubated at room temperature for 1 h. After washing, the reacted antibodies were detected using the HRPO-labeled goat anti-mouse IgG (H + L) and ABTS substrate (Roche Diagnostics). The OD_{405} was recorded and used as a relative measure of antibody titer.

The number of Tat-specific IFN-γ secreting cells indicating specific CTL activity was determined by ELISPOT assay (Takamura et al. 2005). Briefly, a 96-well nitrocellulose plate (Millipore Corporation) was coated with anti-mouse IFN-γ mAb R4-6A2 (Phermingen) and incubated at 4°C overnight. After washing with PBS, complete medium with 10% fetal calf serum was added and incubated at 37°C for 1 h. Triplicate samples of $CD8^+$ T cells separated from the spleen of the immunized mice were plated in two-fold dilutions from 5×10^5 to 6.25×10^4 cells/well [29], added with Tat CTL peptide and incubated for 24 h at 37°C in 5% CO_2. After washing with PBS-T, biotinylated anti-mouse IFN-γ mAb XMG1.2 (Phermingen) was added and incubated overnight at 4°C. Plates washed with PBS-T were added with streptavidin-conjugated alkaline phosphatase (AP) (Mabtech AB) and visualized using AP color development buffer

(BIO-RAD) and counted by KS ELISPOT (Carl Zeiss, Inc.).

Results and discussion

Preferential expression of mTat/Tat-GUS fusion protein in tomato plant

Comparison between the control and transgenic tomato lines (Fig. 2a) showed that the transgenic lines were stunted in growth compared to the control tomato lines consistent with Tat expression (Ramirez et al. 2007; Karasev et al. 2005). Among 82 total calli bombarded (with either Tat or mTat), 55 (67.1%) survived with 14 (25.5%) confirmed plant regeneration. To confirm transformation, tomato extracts were used for Western blot analysis using anti-Tat and anti-M2 antibodies. A ~40 kDa protein, representing the mTat/Tat-GUS fusion protein (mTat/Tat is 14 kDa in size and GUS is 26 kDa in size), was detected instead of the expected 14 kDa size representing an mTat/Tat-only protein (Fig. 2b, c).

The indirect fusion between *mtat/tat* and *gusA* genes was designed to allow the tomato plant to selectively express either a fusion protein, a single protein or both. As seen in Fig. 2b, Tat and mTat were successfully expressed in all tomato extracts but only as a fusion protein and regardless of the stop codon found downstream of the *tat* gene which would have allowed a Tat-only protein to be expressed. Figure 2c illustrates the expected (Tat-only) and actual (Tat-fusion) proteins detected in the transgenic tomato lines. Expression of a fusion protein is suggestive of a codon read-though event (Tork et al. 2004) implying preferential expression of the fusion protein over the Tat-only protein in tomato plants. To resolve the transgenic nature of the Tat- and mTat-bombarded tomato calli, RT-PCR was performed. Figure 2d confirms the active transcription and presence of *mtat/tat* mRNA in all three sets of the transgenic tomato callus, shoot and leaf. In addition, since we were able to amplify a PCR product using a forward

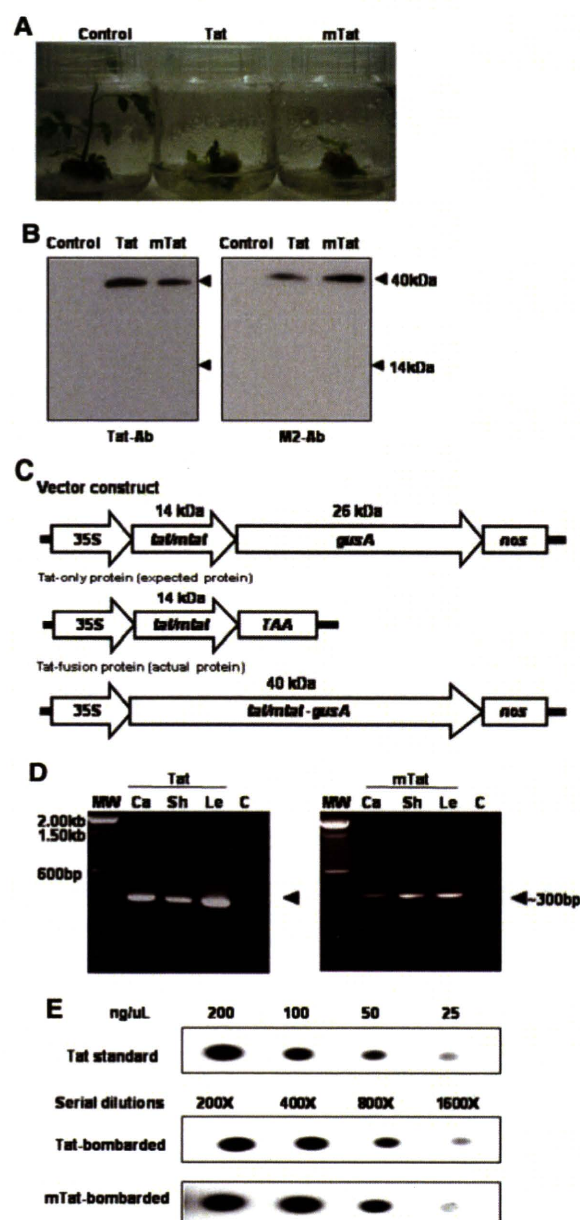

Fig. 2 Expression of both Tat and mTat proteins in tomato plant using particle-gun bombardment. **a** All tomato calli bombarded followed the same bombardment conditions and grown for 3 weeks. Control samples were bombarded with pBI121 vector only. Tat and mTat samples were bombarded with Tat- and mTat-pBI121 vectors, respectively. **b** Western blot assay using tomato extracts from the bombarded samples and detected with anti-Tat and anti-M2 antibodies as indicated in the *bottom*. Only a 40 kDa protein was clearly detected from either Tat- and mTat-pBI121 bombarded tomatoes representing a mTat/Tat-GUS fusion proteins. No 14 kDa protein was detected. **c** Schematic illustration of expected (Tat-only) and actual (Tat-fusion) protein transiently expressed. **d** Reverse transcription-PCR was performed using cDNA obtained from both Tat- and mTat-bombarded callus (*Ca*), shoot (*Sh*) and leaf (*Le*) tissues. Likewise, cDNA obtained from pBI121-bombarded tomato were used as controls. **e** Dot-blot assay providing estimated amounts of Tat expressed in both Tat- and mTat-bombarded tomato plants

primer based on the *tat* gene and a reverse primer based on the *gusA* gene, we show that a *tat-gusA* mRNA is transcribed further confirming production of Tat-GUS fusion protein. Furthermore, tomato extracts were found to contain ~2–4 μg mTat/Tat-GUS fusion protein per milligram plant protein (Fig. 2e) much higher than previous attempts (Ramirez et al. 2007; Karasev et al. 2005). This would imply that in tomato, Tat-GUS fusion protein is the protein form preferentially expressed allowing for a higher amount of protein production. Though the reason that drives the tomato plant to preferentially express the fusion protein is unclear, the significance of both the fusion protein and the amount produced in tomato plant was tested for its immunogenicity by injecting the tomato extracts into Balb/c mice.

Induction of antibody and CTL in Balb/c mice using recombinant tomato extracts

To test the immunogenicity of the fusion protein, Balb/c mice were intradermally injected with the recombinant tomato protein extracts and checked for immunogenic responses. Considering, previous attempts using a Tat-only protein have, thus far, successfully induced a humoral immune response (Ramirez et al. 2007; Karasev et al. 2005), we first established the consistency of humoral immune induction using Tat, in fusion form, in Balb/c mice. Humoral IgG immune responses were detected in the range of 1:10–1:160 titers before leveling-off at 1:320 for both mTat- and Tat-bombarded extracts (Fig. 3). The tomato extracts were found to induce a humoral immune response, regardless of the nominal amount used, and showed that Tat in fusion form could still induce an antibody response consistently with previous works using Tat-only protein (Ramirez et al. 2007; Karasev et al. 2005).

Interestingly, a cellular immune response, though minimal, was also detected. Cellular immune responses as detected by IFN-γ production were modestly induced at 22–24 cells/1 × 10^6 splenocytes using the recombinant tomato protein extracts (Fig. 4). It is noteworthy that Balb/c mice are normally used to test Th2 immune responses which are known to inhibit macrophage activation and instead stimulate antibody production (Mills et al. 2000), explaining the relatively low IFN-γ produced using our tomato extracts. Nevertheless, of greater

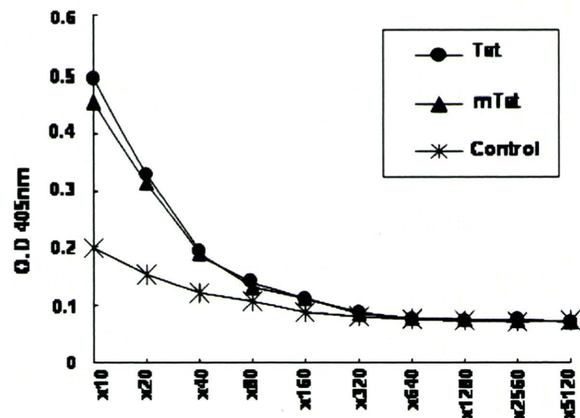

Fig. 3 Induction of IgG humoral immune response in Balb/c mice by recombinant tomato extracts. Plant extracts containing 5 μg Tat were inoculated in five mice intradermally. After 2 weeks, these mice were bled and the anti-Tat IgG antibody titer was measured by ELISA. The figures represent the mean and standard deviation of anti-Tat antibody response but the standard deviations could not be seen because of very small differences. Normal tomato extracts were used as control

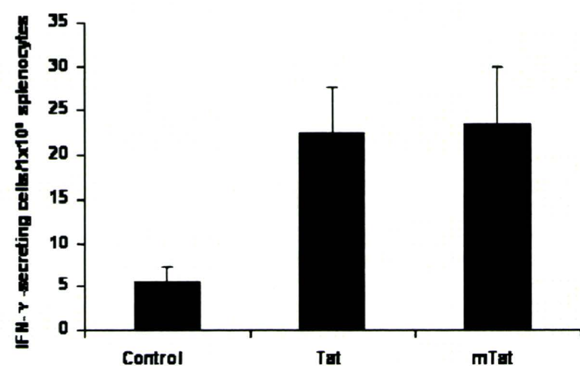

Fig. 4 Induction of cellular immune response in Balb/c mice by recombinant tomato extracts. IFN-γ secreting cells were determined by ELISPOT assay. $CD8^+$ cells were prepared from the spleen of each mouse and approximately 5–6.25 × 10^4 cells per well were incubated with the synthetic Tat peptide (Tat17-25) for 24 h, plates were washed by PBS-T, further incubated overnight at 4°C in the presence of 2 μg/ml of biotinylated anti-mouse IFN-γ monoclonal antibody, and the number of IFN-γ secreting $CD8^+$ T cells were visualized by adding streptavidin conjugated alkaline phosphatases. Data represent the mean and standard deviation of three independent experiments

importance is the ability of Tat protein, in fusion form, to induce a CTL response. The vaccine potential of utilizing HIV-1 Tat relies heavily in its ability to induce both humoral and cellular immunity in the host. Given that both IFN-γ and IgG were detected from the same induced Balb/c mice, it was

clearly demonstrated that a Tat-GUS fusion protein was preferentially expressed over the Tat-only protein in tomato plants. Furthermore, Tat in fusion form would seem to be ideal in inducing both humoral and cellular immune responses which coincidently are the requirements of a model HIV-1 vaccine (Walker and Burton 2008; Gaschen et al. 2002; Borrow et al. 1994). To our knowledge, this is the first report of induction of anti-Tat cellular immunity in Balb/c mice, using Tat protein in fusion form expressed in tomato plant.

No significant difference between wild-type and mutant Tat expressed in tomato plant

It is worth mentioning that tomato plants bombarded with either *mtat* or *tat* gene are both stunted in growth (Fig. 2a) and found to have no significant difference (Fig. 2b, c) implying that the mutations found in mTat are insufficient to distinguish it from Tat when expressed in tomato. With regards to immunogenicity, although previous findings (Kanazawa et al. 2000; Okamoto et al. 2000; Lilen et al. 2002) suggested that wild-type Tat might inhibit immune responses by downregulating the function of antigen presenting cells, the extent of immune responses elicited by either Tat or mTat did not apparently show any difference in mice. This is perhaps because murine Cyclin T1 does not bind to HIV-1 Tat (Bieniasz et al. 1998). Thus, a similar study using primates is warranted since, in human and primate cells, we expect a lower immunogenicity for Tat compared to mTat because of the recruitment of Cyclin T1 that is required for the action of class II transactivator expressing class II MHC molecules (Kanazawa et al. 2000; Okamoto et al. 2000; Lilen et al. 2002).

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Simian Betaretrovirus Infection in a Colony of Cynomolgus Monkeys (*Macaca fascicularis*)

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Of the 419 laboratory-bred cynomolgus macaques (*Macaca fascicularis*) in a breeding colony at our institution, 397 (95%) exhibited antibodies or viral RNA (or both) specific for simian betaretrovirus (SRV) in plasma. Pregnant monkeys (*n* = 95) and their offspring were tested to evaluate maternal–infant infection with SRV. At parturition, the first group of pregnant monkeys (*n* = 76) was antibody-positive but RNA-negative, the second group (*n* = 14 monkeys) was positive for both antibody and RNA, and the last group (*n* = 5) was antibody-negative but RNA-positive. None of the offspring delivered from the 76 antibody-positive/RNA-negative mothers exhibited viremia at birth. Eight of the offspring (including two newborns delivered by caesarian section) from the 14 dually positive mothers exhibited SRV viremia, whereas the remaining 6 newborns from this group were not viremic. All of the offspring (including 2 newborns delivered by caesarian section) of the 5 antibody-negative/RNA-positive mothers exhibited viremia at birth. One neonatal monkey delivered by CS and two naturally delivered monkeys that were viremic at birth remained viremic at 1 to 6 mo of age and lacked SRV antibodies at weaning. Family analysis of 2 viremic mothers revealed that all 7 of their offspring exhibited SRV viremia, 6 of which were also antibody-negative. The present study demonstrates the occurrence of transplacental infection of SRV in viremic dams and infection of SRV in utero to induce immune tolerance in infant monkeys.

Abbreviation: SRV, simian betaretrovirus.

Although simian betaretrovirus (SRV) causes symptoms of immunodeficiency, including anemia, tumors, and persistent refractory diarrhea, in some infected macaques,[1,7,10] most infected monkeys exhibit few or no clinical signs.[2] Macaques free of SRV are important in many types of experiments to avoid associated immunologic and virologic effects. Establishing an SRV-free breeding colony is paramount for a steady supply of appropriate monkeys for various experiments.[8]

We previously reported that SRV-T, a novel subtype of SRV, was found in the cynomolgus colony of our institution.[3] Approximately 20% of the colony monkeys tested in 2005 were viremic and shed SRV-T virus in saliva, urine, and feces.[4,5] The viruses shed by these monkeys are a potential source of horizontal SRV-T infection, as occurred in a rhesus monkey colony.[6,7] In the present study, we investigated the actual prevalence and transmission of SRV in the closed cynomolgus colony through several generations, to prevent the spread of the virus and to establish an SRV-free colony.

Materials and Methods

Animals. The Tsukuba Primate Research Center (Tsukuba, Japan) maintains approximately 1500 cynomolgus monkeys as a breeding and rearing colony and has been maintained as a closed colony for 30 y. All adult monkeys are kept in single cages. Pregnant monkeys are produced by timed mating system in which a female monkey is placed into a male monkey's cage for 3 d; pregnancy is confirmed by ultrasonography 5 wk after mating.

Dams nurse their offspring until weaning at approximately 6 mo. Weaned infants are paired with infants of similar size. Artificial nursing is performed when the dams do not exhibit appropriate nursing behavior.

The housing and care procedures of this study were approved by the Animal Welfare and Animal Care Committee of Tsukuba Primate Research Center of the National Institute of Biomedical Innovation.

Samples. Blood samples were collected from 419 breeders (female, 364; male, 55). All of these monkeys were born at Tsukuba Primate Research Center and are the second and third generations from the founder monkeys, which originated from the Philippines, Malaysia and Indonesia.

We selected 95 pregnant monkeys that exhibited SRV-specific antibodies by Western blotting or the virus as detected by RT-PCR (or both) as the subjects of the study. Blood samples from the mothers and the newborn infant monkeys were collected within 12 h after parturition.

Western blotting. SRV-specific Abs were assessed by Western blotting using SRV-T.[5] Purified virus for this analysis was obtained from the culture supernatant of cloned SRV-T–infected A549 cells by ultracentrifugation through a sucrose gradient; purified viruses were disrupted by 1% SDS for use as antigen in Western blotting. The criterion for a positive reaction was detection of 2 or more virion-specific bands (that is, Gag and Env proteins).

RT-PCR. RNA was extracted from serum of the monkeys (QIAamp Viral RNA Mini Kit, Qiagen, Tokyo, Japan, or MagNA Pure Compact Nucleic Acid Isolation Kit I, Roche, Mannheim,

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Germany). Reverse transcription was performed (ThermoScript RT-PCR System, Invitrogen, Tokyo, Japan) by using gene-specific reverse primers. PCR analysis was performed (Premix Ex*Taq* Hot-Start Version, Takara, Shiga, Japan) by using published sets of external primers (SRVenv1E and SRVenv2E) and nested primers (SRVenv3N and SRVenv4N).[9]

Results

SRV infection status of the 419 laboratory-bred breeders. Of the 419 (female, 364; male, 55) cynomolgus macaques evaluated, 22 were negative for both SRV-specific antibodies and RNA. Of the remaining 397 breeders, 340 were positive for SRV-specific antibodies but were not viremic, 29 were positive for both viral RNA and antibodies, and the remaining 28 monkeys had viremia without antibodies.

SRV infection status of 95 pairs of mothers and offspring at birth. RT-PCR and Western blotting of samples from 95 pairs of mothers and offspring at the time of birth revealed that the dams could be grouped into 1 of 3 categories based on the presence of SRV-specific antibodies and viremia.[2] Among the 95 dams, 76 developed SRV-specific Abs without viremia, 14 had both antibodies and viremia, and the remaining 5 were viremic without SRV-specific antibodies.

None of the offspring of the 76 dams that were antibody-positive but RNA-negative were viremic at birth. Eight infants (including 2 delivered by caesarian section) of the 14 dually positive dams were viremic at birth; the remaining 6 infants of dams in this group were viral RNA-negative. All 5 progeny (including 2 infants delivered by caesarian section) of viremic but antibody-negative dams were viremic at birth.

Plasma SRV-specific antibodies and RNA in viremic newborns during the first 6 mo. We then tested the SRV-specific antibody and RNA status of 3 representative viremic newborns at 1, 2, and 6 mo after birth (Table 1). All 3 of the dams exhibited SRV viremia at delivery, and 2 of them also were positive for SRV-specific antibodies. All 3 infants exhibited SRV-specific RNA at all time points, but none was antibody-positive at weaning.

Family analysis of two representative SRV-viremic dams. The SRV status of all 7 offspring born to 2 representative viremic mothers was verified in 2007. Dam 1319711082 and her 4 offspring (infant 1410311011, born 2003; infant 1420506016, born 2005; infant 1420608031, born 2006; and infant 1420709050, born 2007) all demonstrated SRV RNA in tests performed during 2007. In addition, this dam and her oldest infant (1410311011) were antibody-positive, unlike the 3 youngest siblings. Dam 1319710076 and her 3 offspring (infant 1410408017, born 2004; infant 1410508022, born 2005; and infant 1420701001, born 2007) were all RNA-positive but antibody-negative according to tests performed in 2007.

Discussion

In 2005, we reported that about 20% of the cynomolgus monkeys in the colony at our institution exhibited SRV-T viremia and that virus was present in saliva, urine, and feces from the viremic monkeys.[3-5] Because the virus secreted from these monkeys was a potential source of horizontal SRV-T infection, we performed the current large-scale survey of SRV infections in our laboratory-bred monkeys and assessed the transmission of SRV through the generations represented in the colony.

The present study validated our concerns about vertical and horizontal SRV infections in the colony, because more than 90% of the laboratory-born breeders were positive for SRV-specific antibodies or virus (or both). The rate of viremia in the present study (14%) was smaller than that (20%) in the earlier survey,[5] which involved 49 retired breeders. The rate of viremia in a colony may vary depending on the age distribution of animals and their countries of origin. In particular, we hypothesize that the 28 monkeys that exhibited SRV viremia without specific antibodies are immunotolerant to SRV because of being infected in utero, as is reported to occur in rhesus and pigtailed macaques.[7,12]

To evaluate transplacental maternal–infant transmission of SRV, we tested 95 pairs of mothers and newborns, including 4 infants delivered by caesarean section, by using SRV-specific RT-PCR. The results showed that all monkeys exhibiting SRV-specific antibodies without viremia produced newborns without viremia. However, the transplacental SRV infections observed in infants included 4 newborns delivered by cesarean section from viremic mothers. In pigtailed monkeys, SRV2 was detected in the tissues and amniotic fluid of fetuses and in the blood of newborns delivered from viremic mothers.[12] In other cynomolgus monkeys, SRV was transmitted through transfusion of blood from a viremic donor but not from a nonviremic donor.[13] These findings indicate that SRV viremia of the mother is essential to establishing transplacental infection of the fetus. However, the production of 6 SRV-negative newborns from 14 viremic dams with SRV-specific antibodies may indicate that these antibodies reduced the viral loads in the viremic mothers sufficiently to prevent transplacental infection with SRV. Further investigation to quantify SRV in blood and the occurrence of transplacental infections will resolve this question.

An important issue is whether SRV viremic newborns can convert to a nonviremic state after developing virus-specific antibodies. Three infants born from viremic mothers exhibited viremia, which was maintained at 1, 2, and 6 mo of age, with no antibodies at 6 mo of age. In addition, 7 offspring born from the representative 2 SRV-viremic mothers were all viremic, at ages of 6 mo to 4 y. Pigtailed monkey newborns infected transplacentally with SRV2 maintained a viremic state for 1 y without producing antibodies and harbored proviral DNA in many tissues.[11,12] A newborn rhesus monkey produced from a viremic mother was SRV1-positive within 24 h after birth and was antibody-negative for as long as 6 mo after birth.[7] These findings suggest that cynomolgus infants infected in utero with SRV and born from viremic mothers are immunologically tolerant to the virus and that they then become the source of SRV infection in the colony.

The cynomolgus monkey breeding colony at our institution has been maintained as SPF with regard to B virus, SVV, SIV, STLV1, and measles virus but not SRV. The cage system used during the first 25 y was a two-story type—monkeys were able to touch feces and urine of animals in adjacent cages. In addition, cages were washed with high-pressure water, perhaps helping to spread virus-contaminated waste and increasing the likelihood of horizontal infections. After redesigning the cage system to a single-story type that prevents monkeys from touching fecal and urine waste from another macaque, we anticipate that we will be able to establish an SRV-free colony by introducing SRV nonviremic monkeys into the breeding colony. Furthermore, elimination of viremic dams, which can become a source of transplacental infection, from the breeding colony is critical to establishing an

Table 1. SRV-specific antibodies and RNA in the plasma of viremic newborns during their first 6 mo

				Status of dam at parturition		Status of infant at				
						0 d	1 mo	2 mo	Weaning (approximately 6 mo)	
Infant ID	Method of delivery	Dam ID	Method of nursing	Antibodies	RNA	RNA	RNA	RNA	Antibodies	RNA
1310611144	Caesarean	1210003019	Artificial	+	+	+	+	+	–	+
1410508022	Natural	1319710076	Artificial	–	+	+	+	+	–	+
1420506016	Natural	1319711082	Maternal	+	+	+	+	+	–	+

Testing of infants for SRV-specific antibodies was delayed until weaning because transplacentally transferred maternal antibodies can persist at 2 mo of age.

SRV-free breeding colony. The establishment of an SRV-free cynomolgus breeding colony is paramount for supplying monkeys that are appropriate for many fields of investigation, including vaccine testing, gene therapeutics, organ transplantation, and infectious disease studies.

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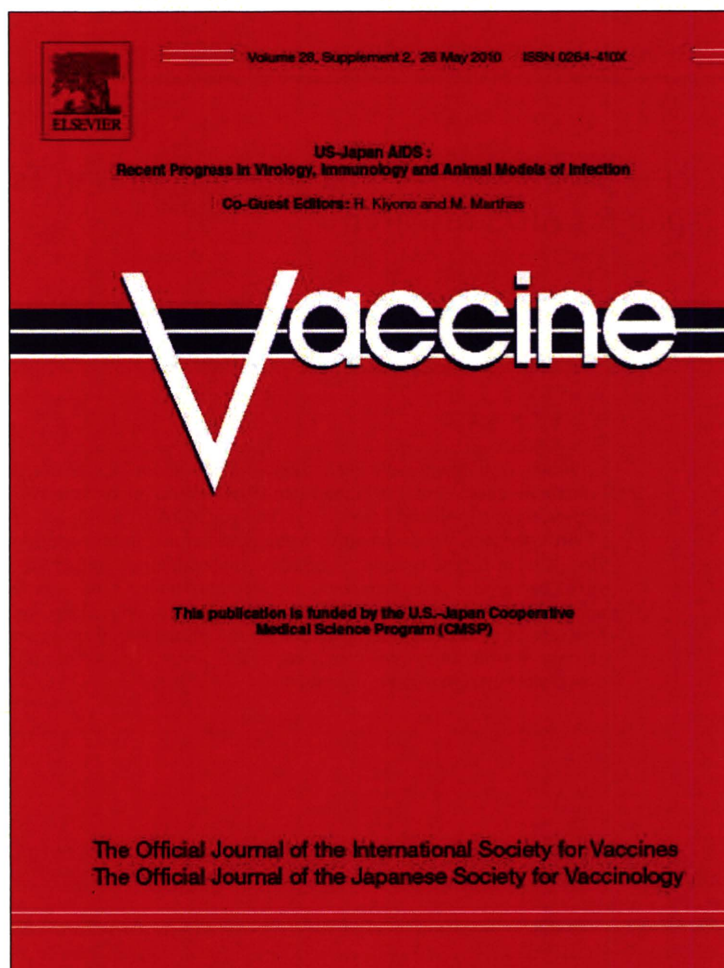

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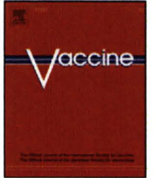

Review

Establishment of specific pathogen-free macaque colonies in Tsukuba Primate Research Center of Japan for AIDS research

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ABSTRACT

Cynomolgus monkeys have been maintained in indoor facilities as closed colony monkeys in Tsukuba Primate Research Center in Japan since 1978. Several microorganisms, including bacteria, parasites and viruses, were eliminated from the cynomolgus monkeys in this colony of TPRC. Various kinds of viruses (B virus, measles virus, simian varicella virus, simian immunodeficiency virus, simian T cell leukemia virus, simian D type retrovirus, simian cytomegalovirus, simian Epstein-Barr virus, and simian foamy virus), bacteria (*Shigella*, *Salmonella* and *Mycobacteria spp.*) and intestinal helminth were chosen as target microorganisms to establish a specific pathogen-free (SPF) colony. Except for a few pathogens (simian D type retrovirus, simian Epstein-Barr virus, and simian foamy virus), selected pathogens were completely eliminated from all monkeys in TPRC. In this review, the history of establishment of SPF cynomolgus monkey colonies in Japan is described.

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1. Introduction

Nonhuman primates are critical resources for biomedical research. Macaque monkeys are one of the key nonhuman primate models that share nearly all characteristics with humans. Conditions of experimental animals are very important for biomedical experiments. The animals should not be infected with microorganisms because microorganism infection may affect results. Moreover, some pathogens are likely to harm not only monkeys but also humans in experiments involving macaques. For these reasons, there is a need for specific pathogen-free (SPF) macaque colonies for research purposes, biohazard avoidance and maintenance of health levels in established colonies (Table 1).

Tsukuba Primate Research Center (TPRC) in Japan has a large-scale breeding colony of experimental cynomolgus monkeys (approximately 1500 monkeys), which play a significant role in the development of pharmaceutical products and medical technologies. The center is the forefront facility in Japan that both supplies laboratory-bred monkeys, mainly cynomolgus monkeys, and performs medical research. Cynomolgus monkeys have been maintained in indoor facilities as closed colony monkeys in TPRC since 1978 [1]. In addition to quality control, supply, research resource development, and basic technology development involving the experimental monkeys, evaluation of state-of-the-art medical technology, evaluation of the efficacy of new drugs and safety assessments are also performed using the monkeys. The establishment of SPF macaques is therefore necessary in TPRC.

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Table 1
History of establishment of SPF cynomolgus monkeys in TPTC.

Year	Target microorganism	Complete elimination from TPRC
1978–1982	BV, MV, *Shigella*, *Salmonella*, *Mycobacteria*, helminth	MV, *Shigella*, *Salmonella*, *Mycobacteria*,
1983–1994	BV, SVV, SIV, STLV-1, SRV/D helminth	SIV, STLV-1, helminth
1995–2004	BV, SVV, SRV/D,	BV, SVV,
2004–Present	SRV/D (73%)[a], LCV (50%)[a], SFV (31%)[a]	CMV

[a] Infection rate of all cynomolgus monkeys in TPRC at present.

The cynomolgus monkeys in TPRC were obtained from Indonesia, Malaysia and Philippines [1]. The monkeys have been bred as pure blood of each origin without interbreed crossing. These pure blood monkeys should be important for comparison of various genetic effects in biological studies including vaccine development. The establishment of SPF colonies in TPRC is also important for this reason. These three pure blood colonies and one mixed blood colony each consist of approximately 100 SPF cynomolgus monkeys. In this review, attempts to establish SPF macaque colonies for advanced biomedical research are reported.

1.1. First term (1978–1882)

Several kinds of microorganisms were chosen for elimination from colony monkeys. Two viruses (B virus and measles virus), three species of bacteria (*Shigella*, *Salmonella* and *Mycobacteria spp.*) and intestinal helminths were selected as the first target pathogens for elimination in macaque colonies. B virus (BV, *Cercopithecine herpesvirus* 1) is an alphaherpesvirus that naturally infects macaque monkeys. In macaques, the virus typically causes a self-limiting disease similar to herepes simplex virus disease in humans [2]. In surprising contrast, BV infection in humans has resulted in the death of 80% of individuals [2]. Therefore, BV was firstly chosen as an SPF target pathogen for prevention of biohazard risks by this virus. The BV infections were detected by BV-specific antibody (Ab) response in sera using an ELISA system (BioReliance Co., USA). Prevention of the spread of BV in the macaque colony was carried out by early weaning of babies from mothers. Infection of the virus in plasma of the prematurely weaned monkeys was confirmed by a BV-specific Ab several times at intervals of 3–6 months. Measles, caused by measles virus (MV) infection, remains a major cause of infant mortality despite the availability of a safe and effective live attenuated virus vaccine. MV-free cynomolgus monkeys are required, since one of the purposes to supply cynomolgus monkeys in TPRC is certification tests for human measles vaccine. MV infection was examined in all monkeys by detection of specific Ab reaction in sera by ELISA and MV antigen (Ag) detected by RT-PCR. Although most of the cynomolgus monkeys from Asia were infected with MV, asymptomatic monkeys with MV excretion in plasma, urine and other biological fluid were not reproduced in TPRC. The MV-infected monkeys were eliminated by this breeding program. Two species of bacteria, *Salmonella* and *Shigella spp.*, were detected by cultivation of rectal or fecal swab samples. Monkeys having these bacteria received drug treatment (200 mg of sulfamethoxazole and 40 mg of trimethoprim once a day for 3 days by oral administration even to *Salmonella*, 200 mg of fosofomycin once a day for 3 days by oral administration even to *Shigella*) if they showed no clinical symptoms of infection with these bacteria. Infection with *Mycobacteria spp.* responsible for tuberculosis was examined by tuberculin (TB) skin tests, and monkeys with positive results of TB skin tests were eliminated. Infection with MV, *Salmonella*, *Shigella* or *Mycobacteria spp.* has not been detected in any monkeys in TPRC since 1982. Cynomolgus monkeys excreting helminth eggs in feces were given anthelimintics (ivermectin 200 μg/kg s.c twice for 2 weeks interval; metronidazol 40 mg/kg once a day for 5 days by oral administration; thiabendazole 50 mg/kg once a day for 3 days by oral administration and mebendazole 20 mg/kg once a day for 3 days by oral administration).

1.2. Second and third terms (1983–1994)

In addition to targeting BV and helminths for elimination from TPRC, simian immunodeficiency virus (SIV), simian T cell leukemia virus (STLV), simian D type retrovirus (SRV/D) and simian varicella virus (SVV) were newly targeted to establish SPF monkey colonies in 1983–1994. Although an AIDS model induced by SIV is very useful for AIDS studies, SIV is not present in macaques from Asia unless they have been experimentally exposed. In fact, natural infection with SIV was not seen in any of the monkeys in TPRC examined by ELISA for detection of SIV-specific Ab in sera. STLV is widely present in all New and Old World primate species. The incidence of STLV infection in most natural simian populations is 5–40%, but it can be much higher in wild monkeys [3,4]. STLV infection was detected in 11.7% of the monkeys in TPRC by IFA using MT-1 cells [5]. These monkeys were eliminated from TPRC over a period of several years. SVV is an alphaherpesvirus that causes varicella in Old World monkeys and establishes latent infection in ganglionic neurons [6]. Outbreaks in many animal facilities have been reported [7]. An outbreak of SVV infection occurred in TPRC during the period from November 1989 to April 1990. Varicella developed in almost 100 monkeys, and 67% of those monkeys died. The rate of infection with SVV in TPRC was 12.9% in 1990. SVV infection can usually be detected by SVV-specific Abs, even in asymptomatic monkeys, and SVV-infected monkeys were eliminated from TPRC in 2000. Attention must be paid to SRV/D both for its risk to macaque colony health and its negative effects on biomedical research. Monkeys infected with SRV/D eventually show symptoms that might be caused by SRV/D infection, such as diarrhea, weight loss and anemia, due to activation attributable to changing conditions of the individual [8–11]. This virus can be transmitted horizontally, vertically or sexually by symptomatic or asymptomatic animals. Moreover, some SRV/D-infected monkeys can become viremic yet remain Ab-negative, allowing infection to escape detection by routine Ab screening [12]. A new subtype of SRV/D, named SRV/D-T, was detected in the colony in TPRC in 2005 [13]. Certain monkeys were found to have plasma viremia of this subtype and did not develop any specific Abs to SRV/D-T. Cynomolgus monkeys in the colony showing SRV/D-T viremia secreted the virus in saliva, urine and feces, and the viruses secreted from these monkeys were thought to be a potential cause of horizontal infections of SRV/D-T. Moreover, there was a high rate of transmission of SRV/D-T infection between mothers and infants in TPRC. Screening for this virus infection was done by detection of both Ab (Western blot analysis) and virus (RT-PCR) in plasma [14]. STLV was completely eliminated from TPRC during the second and third terms.

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1.3. Fourth and fifth terms to present (1995–2009)

Monkey infected with BV and SVV were completely eliminated from TPRC in the late 90s. Three viruses, simian cytomegalovirus (CMV), simian Epstein-Barr virus (EBV, simian lymphocryptoviruses (LCV)) and simian foamy virus (SFV), were added as target viruses in a new plan in 1995 to establish SPF monkey colonies. Simian CMV infections have been reported in various species of monkeys, including macaques [15]. This virus is readily transmitted in oral secretions, breast milk and urine [16], and 3% of adult monkeys in TPRC were infected with the virus. CMV infection was detected by IFA or an ELISA system using CMV Ag. Simian EBV has also been detected in several species of Old World and New World primates [17]. This virus is also readily transmitted, and serological surveys indicated that about 90% of adult cynomolgus monkeys in TPRC were infected. Detection of EBV infection was usually done by using commercial available human IFA kit. Infection with these two viruses, CMV and EBV, in macaques are opportunistic infections. Infection with the other virus, SFV, also does not seem to cause disease in nonhuman primates as natural hosts [18]. Humans can be infected with SFV, although the number of known SFV infection cases in humans is small [19]. SFV infection was detected by IFA using SFV Ag. Monkeys infected with SFV are fraught with hazards to workers in a primate center. The rate of infection with SFV in adult monkeys in TPRC was 80%. Detection of SFV was done by Ab response in sera using ELISA. Prevention of the spread of these three viruses, CMV, LCV and SFV, was performed by artificial nursing with feeding formula for baby monkeys that had been removed from their mothers immediately after birth. CMV infection in monkeys has not been detected in TPRC since 2005.

2. Conclusions

SPF nonhuman primate colonies are required for biomedical research with several beneficial effects such as animal health and occupational safety. High quality of laboratory animals is also required for advanced biomedical studies including vaccine research and development. Infectious agents frequently affect the results of animal experiments. The history of establishment of SPF cynomolgus monkeys in TPRC in Japan for evaluation of state-of-the-art medical technology, evaluation of the efficacy of new drugs and new vaccines, and safety assessments has been described in this review.

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Conflict of interest statement

The author states that they have no conflict of interest.

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—Mini Review—

Cryopreservation of the Ovary

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Abstract: *The removal, cryopreservation, and subsequent reimplantation of ovaries would make it possible to treat a young cancer patient and improve her quality of life by preserving her fertility. The current technology requires cutting the ovary into pieces before freezing and does not support preservation of the whole ovary. The ovary has a complex endocrinologic function. It is composed of cells of different form and character and contains oocytes at various stages of development. Successful cryopreservation, transplantation, and functional rehabilitation of the whole ovary would have broad significance, not only for ovaries but also for other organs such as the liver, kidney, and heart. Ovarian cryopreservation technology would lead the way to the establishment of a biological bank for frozen internal organs.*
Key words: *Cryopreservation, Ovary, Transplantation*

Introduction

Cryopreservation of living cells is an established technology, and the cell banking system provides a source of materials for all aspects of medical research. Clinically, the freezing of mature unfertilized eggs and fertilized eggs has been widely applied to human fertility treatments. What is the difference between freezing ovaries and freezing cells, oocytes, and preimplantation embryos? The difference is the size of the sample. Internal organs are composed of various tissues, and tissues are formed by cells. The egg is the largest cell in the body, but the internal organs are much larger. The size of the sample for freezing affects such factors as temperature change, infiltration of the cryoprotectant, and generation of ice crystals. Recent advances in technology have increased the opportunity to freeze whole ovaries. However, there are still problems to be solved. Here we discuss the current state of the technology for the cryopreservation of whole ovaries.

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Utility of Ovary Freezing

Cryopreservation of the ovary is useful for preserving resources in research using laboratory animals as disease models and for transgenic studies. Clinically, this technology is shifting from the research area to practical uses for maintenance of fertility and improving the quality of life of cancer patients [1 11]. It has even been applied to patients with Turner syndrome, whose ovarian follicles are lost with age. In younger patients, ovary freezing could be used when the vaginal collection of eggs is difficult. At present, human fertility is supported by the use of assisted reproductive techniques such as *in vitro* fertilization and intracytoplasmic sperm injection into mature or immature oocytes. The probability of achieving conception depends on the method of egg collection before the ovary is frozen. Therefore, ovary freezing will expand the range of current applications in the medical technology.

Current State of Ovary Cryopreservation

Ovary cryopreservation was developed using animals [12 16] such as mice and monkeys. Ovary freezing is believed to be a useful means for preserving reproductive cells, but the technology to achieve conception is not yet at the practical stage. The first child to be born from the transplantation of frozen-thawed sliced ovaries was reported in 2004 [3, 4]. Since then, human births resulting from the use of this technology have been widely reported [5 7]. For cancer patients, the ovaries are removed before the

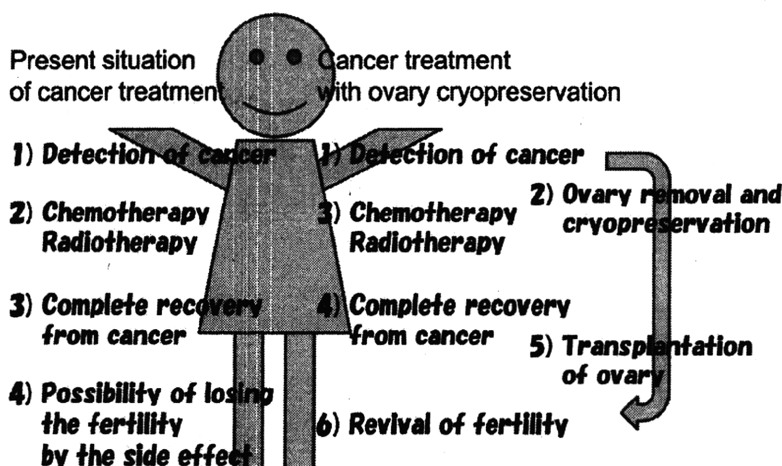

Fig. 1. Example of the application of ovary cryopreservation. The fertility of a cancer patient may be lost due to the side-effects of the treatment of the cancer cells with chemotherapy and radiotherapy (Left side). Before the treatment of the cancer, the ovary can be removed and preserved (Right side).

start of cancer treatment, and the frozen-thawed ovaries are retransplanted to the patient when the cancer has been successfully treated, because there is a possibility that the ovarian germ cells may undergo damage along with the cancer cells during treatment. One of the new cancer treatments preserves the fertility of the patient, but this technique is complicated (Fig. 1).

The current method for ovary freezing involves slowly freezing slices of the ovary. This method was devised to improve the success rate of freezing because the whole ovary is too large to be frozen using current methods. It is common to use a cryoprotectant for cell freezing. By slicing off a large piece of ovary tissue or cutting it into smaller pieces, the effect of the cryoprotectant is known. However, there can be physiological and biological problems with slicing or cutting the ovary. The organ slice begins to lose cells at the contact site of the slice with the screen insert, and thereby reduces organ slice architectural stability and viability. We believe that many problems can be avoided if it were possible to freeze the whole ovary, and into whole ovary preservation has already begun.

Theme for the Future

Thinly slicing the ovary or cutting it into small pieces is an excellent way for the cryoprotectant to infiltrate the specimen and for an even temperature to be maintained. However, the number of ovarian follicles that exist in a cut ovary (10 mm × 10 mm × 1 mm) is thought to be limited, and many of them can be lost during the process of freezing, thawing and transplantation. It seems that small ovarian follicles (such as a primordial ovarian follicle) can survive. Therefore, it is currently necessary to wait for several months to confirm the functionality of a transplanted ovary. Furthermore, the long-term maintenance of ovarian function cannot be guaranteed. It is necessary to analyze in detail the living cells in the thawed ovary. Cells (oocytes) at various stages and cells with endocrinologic function exist in the ovary. The state of each cell cannot always be determined. Moreover, it will be necessary to verify the effect of cryoprotectant and its side-effects after transplantation.

It is not known why a transplanted ovary sometimes does not function. Therefore, the problems of cryopreservation cannot be solved by focusing on the freezing technology alone. It is also necessary to perfect the thawing method. In the thawing method, it is extremely difficult to thaw both the inside and the outside of large internal organs under the same conditions. It is difficult to achieve success according to the fundamental principles of physics in freezing theory, even though the theory has been clarified [17 23]. When one does not achieve an excellent result by analyzing a frozen-thawed organ transplantation sample, it should be considered that there should be problems in both the freezing and the thawing

technology. Thus, the further development of thawing technology is an important area of research.

Moreover, the implantation technique cannot be disregarded. It is necessary to make blood circulate within the ovary several hours or a few days after transplantation of the ovary. If the nutritional content of the cells is not supplied with blood, an individual cell cannot survive at body temperature. Ideally, each ovary is frozen with a blood vessel, and the blood vessel is sutured during transplantation. We are attempting to improve the method of cryopreservation of blood vessels to solve this problem.

In one clinical application of cryopreservation, the ovaries of female cancer patients are removed and frozen before cancer treatment begins, and then the ovary is transplanted after the cancer has been successfully treated. There will be no cancer cells in the ovaries, but because of the possibility of minimal residual disease (MRD), it is necessary to assess the transplant carefully. A new technology for MRD detection needs to be developed [24, 25]. Furthermore, an ovum, embryo or ovary should be selected for cryopreservation according to the patient's age and the degree of damage to the ovary, because of the close relationship between the age of a woman and the degree of ovarian function.

It is necessary to select the appropriate treatment for each patient who desires a natural pregnancy, such as assisted reproductive techniques involving ovary removal on only one side, whole ovary removal, or partial excision. Moreover, data should be accumulated about each technique to improve the survival rate of the ovary after transplantation and the timing of retransplantation to maintain ovarian function at the site of the graft.

Upgrade of Technology and the Importance of Education

Theoretically, it is effective to freeze the whole ovary and to keep the ovary function long time when transplanting an ovary. If the loss of ovarian follicles can be reduced to the minimum, ovarian function should be maintained for a long time. To achieve this, it is necessary to develop freezing and thawing technologies for the whole ovary and to solve the problem of thrombosis during vascular anastomosis. The rate of pregnancy after such procedures is extremely low, although pregnancies have been reported in animals. However, live births after transplantation of the whole ovary have not been reported in humans [26].

Cryoprotectants cannot work properly on the whole ovary. However, the damage from ice crystals in cells can be reduced by slightly magnetizing the whole ovary during freezing, without a cryoprotectant. The following conclusions are possible: (i) a cryoprotectant is not needed, (ii) thrombosis can be prevented, and (iii) large growing follicles can survive. If this magnetization technique could be established, it would be possible to apply it not only to the ovary but also to other internal organs. This technology may even be useful in the field of organ transplantation.

Although research on the cryopreservation of internal organs started only recently, this technology should improve the ability to treat cancer in young patients and their quality of life after recovery [27, 28]. Medical researchers and physicians in various fields should cooperate in the development of this technology. We strongly encourage supplying accurate information about the technology not only to medical personnel but also to the general public.

Possible Applications of Cryopreservation Technology in Other Fields, and Future Perspectives

The most important goal of cryopreservation technology is to maintain the function of the cell after freeze-thawing. The ovary is one of the sources of female reproductive cells and has an endocrinologic function. In other words, it is a complex internal organ that contains cells of various kinds and at various stages of development. Once the freezing of the ovary becomes feasible and the return to ovarian function after transplantation is certain, it may also be possible to freeze other internal organs such as the heart, liver, and kidneys. As we know that the cell-mediated immune response molecule, the major histocompatibility complex (MHC), varies greatly between individuals and mismatch of MHC antigen is an important factor in the acute rejection of the transplanted tissues. If an information bank of MHC antigen for frozen internal organs used in organ transplantation can be successfully developed, medical treatment with organ transplants could be greatly improved, since it is thought that a frozen internal organ banking system could serve the entire world (Fig. 2). In addition, the utility of the umbilical funiculus, including stem cells, is parallel to that of bone marrow stem cells. If freezing the umbilical funiculus were to become possible, the number of stem cells preserved would increase. Technological development might also enable the cryopreservation of blood.

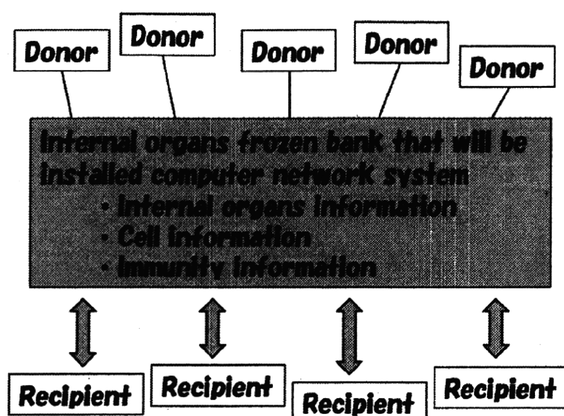

Fig. 2. Schematic diagram of an internal organ cryobank. Time can be saved in the transportation of internal organs and the matching of the donor and the recipient. The establishment of an organ cryobank would help to meet many of the challenges faced in transplantation of an organ between a donor and a recipient living elsewhere in the world.

Ovary cryopreservation technology contributes greatly to both laboratory animal research and clinical applications. Further advancement of organ cryopreservation technology is expected to occur through research into the freezing of different organs in the near future.

Conclusion

The technology of ovary cryopreservation has progressed and has opened many possible doors. This technology will give hope to young women with cancer. Moreover, existing research suggests the possibility of constructing a bank for frozen internal organs.

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