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—Note—

Female Reproduction Characteristics in a Large-Scale Breeding Colony of Cynomolgus Monkeys (*Macaca fascicularis*)

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Abstract: We studied two mating systems for a large-scale breeding colony of cynomolgus monkeys: (i) a 3-day timed system that keeps one female and one male together in a cage for 3 days beginning 11 days after menstruation, and (ii) a 7-day timed system beginning 9 days after menstruation. Regardless of the mating system used, we propose a practical definition of an infertile female as one that does not become pregnant in up to six pairings. If we eliminate these animals from the breeding colony, the pregnancy rates would be 3.6 times higher. Eliminating infertile females from a breeding colony is important in order to save labor, time and cost and to maintain a healthy breeding colony.

Key words: cynomolgus monkey, menstrual cycle, reproduction

Since the establishment of the Tsukuba Primate Research Center in 1978, we have been working to develop a breeding system for successive generation of cynomolgus monkeys (*Macaca fascicularis*), and a rearing system for high-quality production of primates, such as specific pathogen-free animals [2], for use in scientific experiments. Laboratory-bred cynomolgus monkeys are widely used as the preferred model among primate species, and therefore an efficient breeding system is required. Although a group breeding system consisting of several females and one male—the so-called harem system—is commonly used, it has some drawbacks in that it is impossible to determine the exact ages of fetuses or expected dates of birth. A mating system in which one female and one male are joined together (pairing) in a cage on the expected day of ovulation makes it easier to estimate ages and dates of birth; we have been using this system for 30 years [3, 4]. However, this system requires knowledge of ovulation day, which depends on the length of the female's menstrual cycle [6]. Previous cycle lengths are usually used for predicting the following pairing period, even in our breeding colony. However, a marked increase in serum follicle stimulation hormone (FSH) concentration is observed from 8 to 15 days after menstruation with no relation to menstrual cycle length; in 60% of animals observed, FSH increases at 10 to 11 days after menstruation, and ovulation is observed 1 or 2 days later. Therefore, the length of the previous menstrual cycle may not help in predicting the length of the next cycle [9], and the opti-

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mal mating time is judged to be about 12 days after menstruation regardless of the menstrual cycle length [5, 7].

We wished to demonstrate the usefulness of a simplified mating system which doesn't require the previous menstrual cycle lengths. In addition, to determine the breeding efficiencies, we compared a 3-day timed mating system, which keeps one female and one male together in a cage for 3 days beginning 11 days after menstruation, with a 7-day timed mating system, which keeps females and males together for 7 days beginning 9 days after menstruation.

We also wanted to elucidate the relation between pregnancies and the number of pairings necessary for each female to become pregnant. We propose a practical definition of the loss of fertility to eliminate infertile animals from a breeding colony to improve the breeding efficiency.

Protocols for all experiments involving animals in this study were in compliance with the guidelines set by the National Institutes of Biomedical Innovation for the care, use and biological hazard countermeasures of laboratory animals. All animals used were laboratory bred and reared at our primate center and were housed indoors in individual cages (80 cm wide × 80 cm high × 85 cm deep; stainless steel mesh) at a room temperature of 25 ± 2°C, relative humidity of 60 ± 5%, a light-and-dark cycle of 12 h light, 12 h dark, and fresh air provided 10 times per hour. Animals were fed daily with ~70 g of commercially prepared monkey chow (Type AS, Oriental Yeast, Tokyo, Japan) and ~100 g of apples. Details of the breeding and rearing conditions of the animals in our breeding colony are described elsewhere [3, 4].

Data were collected from March 2006 to March 2007 and analyzed statistically by the chi-square test.

In this study, 457 females, aged 4–24 years, and 65 males, aged 6–25 years, were used. For the pairing, two individual cages were connected. According to the menstrual bleeding record, a suitable female and a sexually mature male (~4 kg body weight, ~5 years of age and not kin to this female) were kept together in a connected cage (pairing). No other considerations for pairing specific males and females were made. Ultrasonography was conducted 35 days after the first day of pairing [1], and pregnancy was diagnosed by the presence of a gestational sac in the uterus.

Table 1. Relationship between age of females and pregnancy rate in two pairing systems (number of pregnant/number of pairing)

Age	3-day system		7-day system	
	Observed pregnancies	Pregnancy rate	Observed pregnancies	Pregnancy rate
20–24	0/21	0	0/17	0
19	0/13	0	1/2	0.500
18	3/4	0.750	3/8	0.375
17	3/45	0.067	5/26	0.192
16	4/20	0.200	2/30	0.067
15	9/37	0.243	6/46	0.122
14	9/57	0.158	4/34	0.118
13	9/48	0.188	6/36	0.167
12	5/26	0.192	8/37	0.216
11	7/33	0.212	6/32	0.188
10	6/36	0.167	6/34	0.176
9	7/43	0.163	15/44	0.341
8	4/24	0.167	6/32	0.188
7	2/46	0.042	11/56	0.196
6	5/44	0.114	12/48	0.250
5	2/43	0.047	1/47	0.021
4	4/57	0.070	4/43	0.093
Total	78/597	0.131*	95/575	0.165

*No-significant difference between two systems ($P>0.05$).

The pregnancy rate (number of pregnancies/number of pairings) was 0.150, and there was no significant difference between the two mating systems (3-day timed: 0.131; 7-day timed: 0.165; $P>0.05$). The relationship between the age of the females and pregnancy rates is shown in Table 1. Pregnancy was achieved in females >4 years of age but not in those >20 years of age.

The number of pairings necessary for the females to become pregnant is shown in Table 2. Half of the pregnancies resulted from the first pairing, regardless of the mating system used (3-day timed: 41 of 78 cases [0.53]; 7-day timed: 55 of 95 cases [0.58]) or the age of the female. Eleven animals <5 years of age were included in this group.

Fifteen animals failed to become pregnant after six pairings and were judged to have lost their fertility. Animals that were judged to be infertile at the end of the observation period had had their first delivery at 5.7 ± 1.2 (mean ± SD; range, 4–8) years of age and their last delivery at 20.0 ± 1.8 (range, 17–22) years of age; the total number of deliveries in their productive span was

Table 2. Number of pairings needed for pregnancy initiation

Pairing	3-day system	7-day system
1st	41 (0.53)	55 (0.58)
2nd	17 (0.22)	21 (0.21)
3rd	10 (0.13)	13 (0.13)
4th	5 (0.06)	4 (0.04)
5th	5 (0.06)	0 (0.00)
6th	0 (0.00)	2 (0.02)
Total	78	95

No pregnancy from over 6th to 10th (n=15) pairings was observed.

9.9 ± 1.8 (range, 7 to 13).

Details of experiments in primate species under controlled conditions are rarely published; therefore, it is difficult to compare our data with other data. Published descriptions of large-scale breeding colonies seen even rarer [5, 7, 8].

When calculating the pregnancy rates, we excluded the infertile animals that failed to become pregnant in up to six pairings. The corrected pregnancy rate was calculated by the following formula:

(Total number of pregnancies) ÷ [Sum (1–6) {number of pairings needed to become pregnant (1–6)} × {number of pregnant animals}]

The corrected pregnancy rate in the 3-day mating system was

$78 \div (1 \times 41 + 2 \times 17 + 3 \times 10 + 4 \times 5 + 5 \times 5 + 6 \times 0) = 78 \div 150 = 0.52.$

The corrected pregnancy rate in the 7-day mating system was

$95 \div (1 \times 55 + 2 \times 21 + 3 \times 13 + 4 \times 4 + 5 \times 0 + 6 \times 2) = 95 \div 168 = 0.57.$

There was no significant difference between the corrected pregnancy rates of the two systems; the mean corrected pregnancy rate was 0.54.

The probability of fertile animals becoming pregnant at the sixth pairing was 0.025 (= 0.54 ∧ 6); almost all fertile females (97.5%) became pregnant before that time. Therefore, females that failed to become pregnant by the time of the sixth pairing were judged to have lost their fertility. The probability of fertile animals being misjudged to be infertile was <5% in this breeding system; this value is considered to be acceptable for the management of a large-scale breeding colony.

Table 3. Relationship between age of females and number of pairings for pregnancy (combined data of the two pairing systems, mean ± SD)

Age	Pairings	Animals examined
20–24	–	12
19	1*	1
18	1.5 ± 1.2	6
17	1.5 ± 0.9	8
16	2.7 ± 2.3	6
15	1.8 ± 0.9	16
14	1.9 ± 1.3	13
13	1.6 ± 1.0	15
12	1.4 ± 0.5	13
11	2.2 ± 1.2	13
10	1.8 ± 1.1	13
9	1.7 ± 1.0	22
8	2.2 ± 1.4	10
7	1.7 ± 0.9	13
6	2.1 ± 1.4	16
5	1, 2, 4*	3
4	1.5 ± 1.4	8

* Raw data shown.

The relationship between the age of the female and the number of pairings necessary to become pregnant is shown in Table 3. As no significant difference between the two mating systems was observed, the two sets of data were combined. No significant relationship between females 4 to 19 years of age and the number of pairings necessary to become pregnant was observed. No pregnancies were achieved in females >20 years of age.

The relationship between the age of females and the number of pregnancies per female is shown in Table 4. Again, the two sets of data from the two mating systems were combined. There was no significant difference in pregnancy rates (number of pregnancies/number of females examined in each group) in females 6 to 19 years of age (Table 4). However, 4- and 5-year-old animals had much lower rates.

In this study, we wanted to analyze the breeding data from our large-scale breeding colony of cynomolgus monkeys in terms of the females. A prolonged pairing of one female and one male was expected to increase the pregnancy rate. However, in a total of 1,172 pairings involving 457 females, there was no significant difference in pregnancy rates between the 3- and the 7-day

Table 4. Relationship between age and pregnancy (conbined data of the two pairing systems)

Age	Pairings	Animals examined	Pregnancy rate
20–24	0	12	0.00
19	1	5	0.20
18	4	5	0.80
17	8	22	0.36
16	6	14	0.43
15	16	30	0.53
14	13	28	0.46
13	15	33	0.45
12	13	26	0.54
11	13	27	0.48
10	12	23	0.52
9	20	37	0.54
8	10	23	0.43
7	13	40	0.33
6	16	44	0.36
5	3	35	0.09
4	8	51	0.16
Total	171	455	0.38

timed mating systems (Table 1). These results are in agreement with those of previous reports [7, 8]. No significant differences between the two different mating systems were observed in this study. This may be due to the fact that copulatory behavior is often observed early in the pairing period (unpublished data), so prolonged paring may not have had an effect on the reproductive parameters observed in this study. Furthermore, females >20 years of age (a total of 38 pairings involving 12 females) did not become pregnant, and these females were judged to have lost their fertility. Although the females <6 years of age had regular menstrual cycles, their pregnancy rates were lower than those of the older animals. These data indicate that the younger animals had not completely reached sexual maturation.

One half of the pregnancies were achieved after the first pairing, regardless of the mating system used or the age of the female (Table 2). Pregnancy rates gradually decreased with increasing numbers of pairings, and no pregnancies were achieved after the sixth pairing. We propose the practical definition that animals that do not become pregnant in six pairings or less are no longer fertile. The reason for this phenomenon is unclear. Additional studies of reproductive physiology and behavior are needed to elucidate this. Were we to eliminate infertile animals from the breeding colony, the pregnancy rate would increased 3.6 times, from 0.15 to 0.54, indicating management of infertile females would result in savings in labor, time and the cost of maintaining the breeding colony.

After excluding data from infertile females, no significant differences in reproductive ability, such as the number of pairings necessary to become pregnant (Table 3) or the number of pregnancies per 6- to 19-year-old female (Table 4), were observed. The usefulness of the 3-day mating system in a large-scale breeding colony, regardless of the length of the menstrual cycle in each female, has thus been demonstrated. Further analysis of data regarding males in a large-scale breeding colony is still needed.

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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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REPRODUCTION
RESEARCH

Characterization of a novel embryonic stem cell line from an ICSI-derived blastocyst in the African green monkey

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Abstract

Several cell types from the African green monkey (*Cercopithecus aethiops*), such as red blood cells, primary culture cells from kidney, and the Vero cell line, are valuable sources for biomedical research and testing. Embryonic stem (ES) cells that are established from blastocysts have pluripotency to differentiate into these and other types of cells. We examined an *in vitro* culture system of zygotes produced by ICSI in African green monkeys and attempted to establish ES cells. Culturing with and without a mouse embryonic fibroblast (MEF) cell monolayer resulted in the development of ICSI-derived zygotes to the blastocyst stage, while culturing with a buffalo rat liver cell monolayer yielded no development (3/14, 21.4% and 6/31, 19.4% vs 0/23, 0% respectively; $P<0.05$). One of the nine blastocysts, which had been one of the zygotes co-cultured with MEF cells, formed flat colonies consisting of cells with large nuclei, similar to other primate ES cell lines. The African green monkey ES (AgMES) cells expressed pluripotency markers, formed teratomas consisting of three embryonic germ layer tissues, and had a normal chromosome number. Furthermore, expression of the germ cell markers *CD9* and *DPPA3* (*STELLA*) was detected in the embryoid bodies, suggesting that AgMES cells might have the potential ability to differentiate into germ cells. The results suggested that MEF cells greatly affected the quality of the inner cell mass of the blastocysts. In addition, AgMES cells would be a precious resource for biomedical research such as other primate ES cell lines.

Reproduction (2010) **139** 565–573

Introduction

In vitro culturing is an important technique for effectively producing individuals from embryos that were manipulated *in vitro*. However, the *in vitro* culturing for the African green monkey (*Cercopithecus aethiops*) embryos has not been examined. In rhesus monkey embryos, co-culture with a buffalo rat liver (BRL) cell monolayer in CMRL-1066 has been utilized (Zhang *et al.* 1994, Nusser *et al.* 2001). On the other hand, mouse embryonic fibroblast (MEF) cells have been widely utilized for the establishment of embryonic stem (ES) cells, showing that MEF cells affect establishment of the ES cells from the inner cell mass (ICM), where develop to an individual in the future, of blastocyst. We therefore considered that MEF might also enhance the growth of African green monkey embryos cultured *in vitro*.

Primate ES cell lines were established in the rhesus monkey (*Macaca mulatta*) for the first time in 1995 (Thomson *et al.* 1995) and were subsequently established in the common marmoset (*Callithrix jacchus*) in 1996 (Thomson *et al.* 1996), in humans in 1998 (Thomson *et al.* 1998), and in the cynomolgus monkey (*Macaca fascicularis*) in 2001 (Suemori *et al.* 2001), aiming at utilization in biomedical research. In particular, application to regenerative medicine is expected. For this purpose, methods to induce ES cells to develop into specific differentiated cells have been examined, based on the pluripotency of ES cells, as well as their self-renewal and stable karyotype characteristics, which are important properties for not only regenerative or transplantation medicines but also research or inspection at the cytological level.

The red blood cells and primary culture cells from kidney of the African green monkey have been used for the inspection of measles infection and human polio vaccine production respectively. In addition, the Vero cell line established from the kidney of the monkey has been used to test viral infections and to introduce foreign genes. Furthermore, spontaneous systemic amyloidosis was

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Table 1 Attempt to establish embryonic stem (ES) cell lines in African green monkeys.

	Culture of zygotes	Number of blastocysts	Treatment of blastocysts[a]	Number of outgrowths	Passage methods	Colony appearance	Passage methods	Number of ES cell lines established
I	With MEF monolayer	1	Removal of ZP and TE	1	Enzyme and needles	1	Enzyme and needles	1
II		2	Removal of ZP	2	Enzyme and needles	1	Enzyme and pipetting	0
III	Without cell monolayer	3	Removal of ZP and TE	1	Enzyme and pipetting	1	Enzyme and pipetting	0
IV		3	Removal of ZP	0	–	–	–	–

[a]ZP, zona pellucida; TE, trophectoderm.

recently reported in this monkey (Nakamura *et al.* 2008). The African green monkey and the differentiated cells derived from African green monkey ES (AgMES) cells may help to elucidate the mechanism of amyloidosis in humans and contribute to the treatment. Those unique characteristics show that the cells of the monkey are very valuable for biomedical research and testing.

ES cells may be useful as a source of cells with the same genetic background, even if we do not collect them from living individuals. In short, we could reduce the number of monkey needed to supply cells. Here, we report an *in vitro* culture system of ICSI-derived zygotes and the establishment of a novel ES cell line in the African green monkey. We believe that AgMES cells would be a precious resource for biomedical research in addition to other primate ES cell lines.

Results

In vitro *culture of ICSI-derived embryos*

Blastocyst stage embryos were prepared from zygotes produced by ICSI as described in our previous report (Shimozawa *et al.* 2007). A total of 68 zygotes with two pronuclei and a second polar body were cultured with or

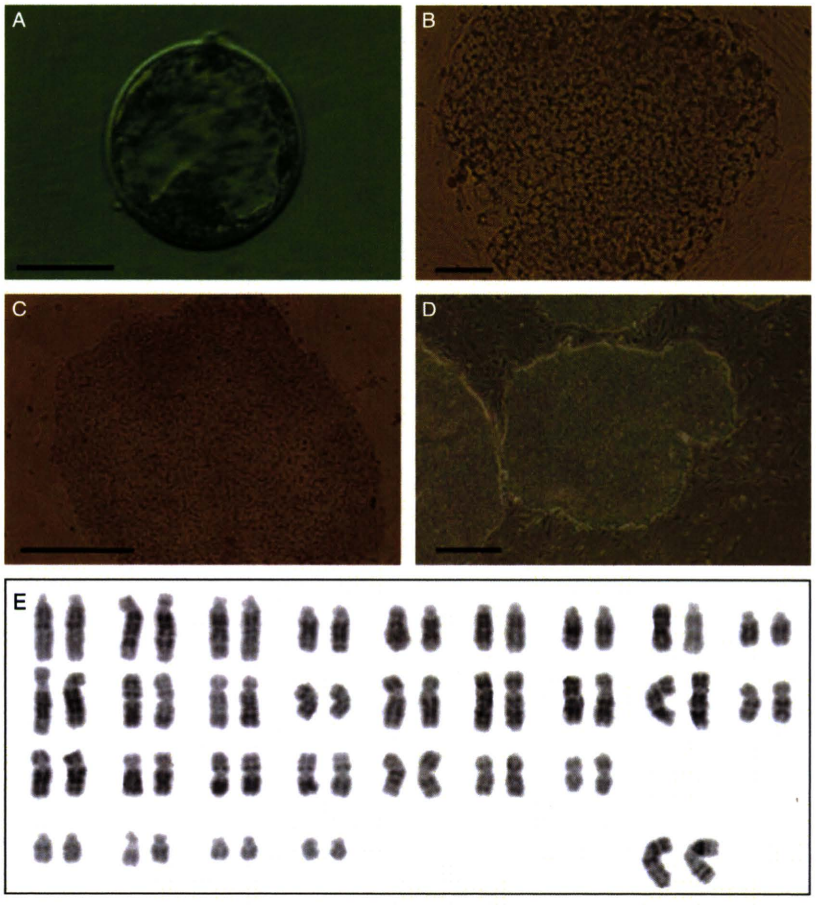

Figure 1 Establishment of the AgMES cell line. Parts of the inner cell mass isolated from an ICSI-derived blastocyst (A) were transferred onto a mitomycin C-treated MEF cell monolayer, which then formed an outgrowth (B). At the first passage dividing the outgrowth into small clusters with collagenase and needles, a new colony appeared (C) and was passaged further. The colonies formed multiple new colonies (D). Hereafter, these colonies were regularly passaged with collagenase and pipetting. Karyotyping analysis revealed that cells examined at passage 19 had a normal chromosome number of 60 and sex chromosomes of XX (E). Bar represents 100 μm (A and B) and 500 μm (C and D).

without BRL cell monolayer or MEF cell monolayer, and then nine of those developed to the blastocyst stage. Culturing with and without the MEF cell monolayer yielded development to the blastocyst stage, while culturing with the BRL cell monolayer yielded no development (3/14, 21.4% and 6/31, 19.4% vs 0/23, 0% respectively; $P<0.05$).

Establishment of ES cells

Nine blastocysts were used to establish ES cells (Table 1). Parts of the ICM isolated by removing the trophectoderm (TE) and the zona pellucida (ZP) with needles (one blastocyst cultured with MEF, hereafter designated I, Fig. 1A; and three without MEF, designated III) or the blastocysts in which the ZP was dissolved with an enzyme (two blastocysts with MEF, designated II; and three without MEF, designated IV) were transferred to ES cell culture medium (ESM) with mitomycin C-treated STO cell or MEF cell monolayer. Of nine blastocysts, four formed outgrowths (one of I blastocysts: Fig. 1B, two of II, and one of III). At the first passage using collagenase and pipetting or dividing into small clusters with needles, three colonies from three outgrowths (one each of I: Fig. 1C, III and IV) appeared and passaged further. The colonies from I passaged with collagenase and needles newly formed multiple colonies (Fig. 1D), but the other colonies passaged with collagenase and pipetting disappeared. Thereafter, these colonies from I were regularly passaged with collagenase and pipetting, and showed flat-formed colony morphology consisting of cells with large nuclei, similar to other primate ES cell lines.

Characterization of undifferentiated ES cells

We examined karyotype and the expression of undifferentiated markers in the AgMES cell line established. Karyotyping analysis revealed that 88% (88/100) of cells examined at passage 19 had a normal chromosome number of 60 and sex chromosomes of XX (Fig. 1E). Immunofluoresence revealed that this cell line strongly expressed POU5F1 (Oct-3), TRA-1-60, TRA-1-81, and NANOG, but not SSEA1 (Fig. 2A–E), and showed alkaline phosphatase activity (Fig. 2F). The expression of SSEA3 and SSEA4 was vaguely observed. Furthermore, gene expression analysis of the pluripotency markers by RT-PCR demonstrated that *POU5F1* (*OCT3/4*), *NANOG*, *SOX2*, and *REX1* were present in AgMES cells, but *FOXD3* was not, although three primer sets for *FOXD3* were used (Fig. 2G). On the other hand, in CMK6 (AGC Techno Glass Co., Ltd, Chiba, Japan),

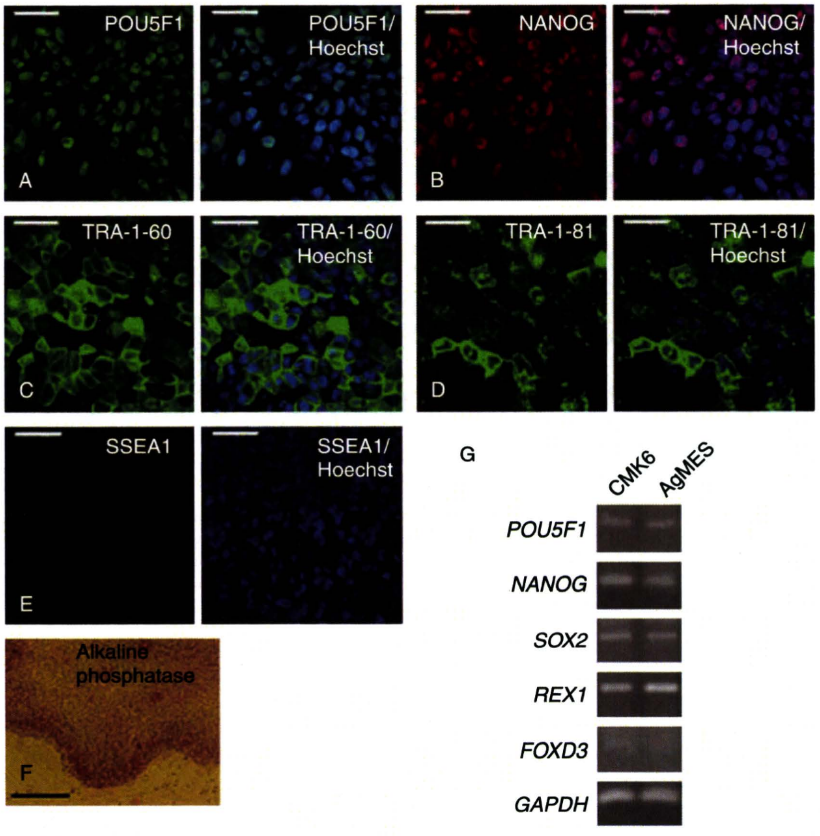

Figure 2 Characterization of undifferentiated AgMES cells. AgMES cells were stained with antibodies against pluripotency markers and alkaline phosphatase (A–F). POU5F1 (A), NANOG (B), TRA-1-60 (C), TRA-1-81 (D), SSEA1 (E), and alkaline phosphatase (F). Nuclei were counterstained with Hoechst 33342 (blue) (A–E). A double staining with POU5F1 (A) and NANOG (B) was conducted in the same specimen. Bar represents 50 μm (A–E) and 200 μm (F). Gene expression analysis of pluripotency markers by RT-PCR in AgMES cells was conducted (G). The results were compared with those for CMK6, a cynomolgus monkey ES cell line established by Suemori *et al.* (2001), as a control.

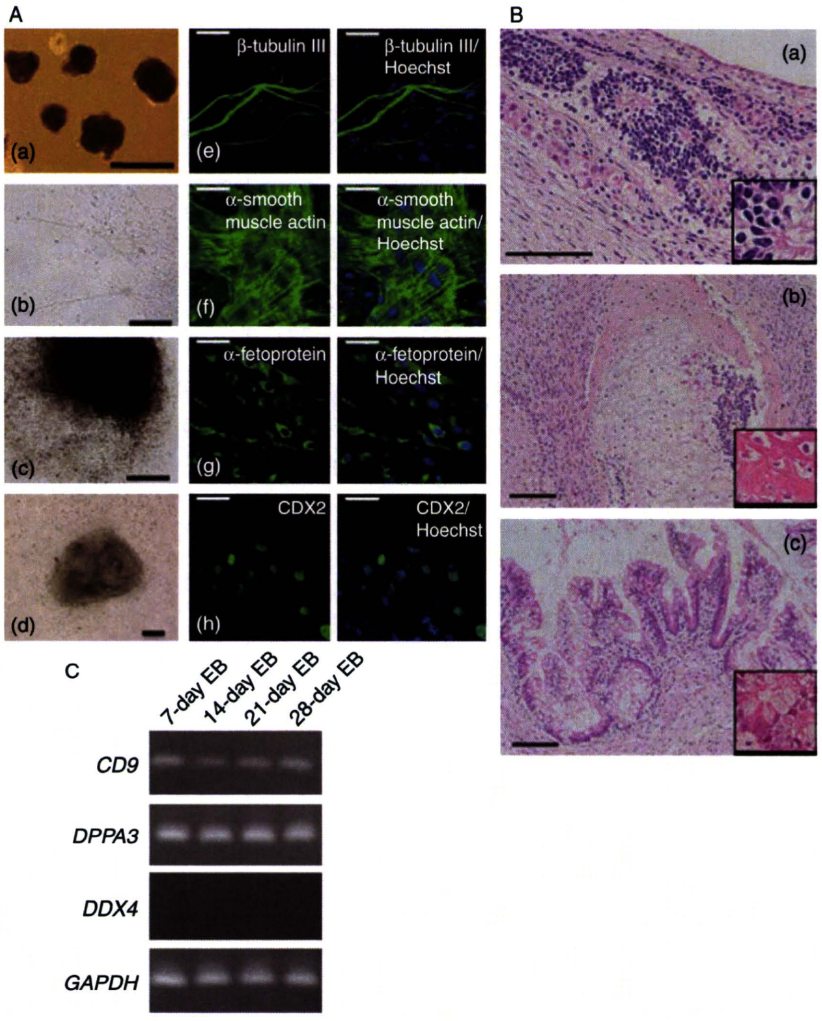

Figure 3 Characterization of derivatives from AgMES cells. (A) EBs (a) at 2 weeks after unattached culture spontaneously differentiated to neuron-like cells (b), pigment cells (c), and myocardial-like cells with beating clusters (d). In the derivatives, the expression of β-tubulin III (e, ectoderm), α-smooth muscle actin (f, mesoderm), α-fetoprotein (g, endoderm), and CDX2 (h, trophectoderm) was confirmed. Nuclei were counterstained with Hoechst 33342 (blue) (e–h). Bar represents 250 μm (a–d) and 50 μm (e–h). (B) Histological analysis of teratomas formed from AgMES cells. Characteristic morphologies; neural (ectoderm, a), osseous (mesoderm, b), and gut tissues (endoderm, c) were confirmed. Each figure includes insets for showing high magnification of typical cells, such as neuroblasts (a), osteoblasts with osseous matrix (b), and goblet cells (c). Bars represent 100 μm, 40 μm (inset a), and 20 μm (inset b and c). (C) Gene expression analysis of germ cell markers by RT-PCR in EBs at days 7, 14, 21, and 28 of culture.

the cynomolgus monkey ES cell line established by Suemori *et al.* (2001) and employed here as a control, the expression of all markers examined was detected (Fig. 2G).

Pluripotency analysis

To examine the pluripotency of AgMES cells, we induced the development of embryoid bodies (EBs) and teratomas *in vitro* and *in vivo* respectively. EBs were developed by the floating culture of ES cell colonies recovered with collagenase treatment (Fig. 3A; a). After 2 weeks of culturing, most EBs formed solid type clusters. EBs were transferred to tissue culture dishes and then attached. Attached EBs showed outgrowths and spontaneously formed the various differentiated cells such as neuron-like cells, pigment cells, and beating myocardial-like cells (Fig. 3A; b–d). These spontaneous differentiated cells from EBs showed the expression of β-tubulin III (ectoderm), α-smooth muscle actin (mesoderm), α-fetoprotein (endoderm), and CDX2 (TE; Fig. 3A; e–h). Histological analysis revealed that the ES cells transferred into two immunodeficient mice formed teratomas consisting of neural and dermal tissues as ectoderm (Fig. 3B-a; neural tissue), smooth muscle, osseous, cartilage, fatty, fibrous, and vascular tissues as mesoderm (Fig. 3B-c; osseous tissue), and gut tissue as endoderm tissue (Fig. 3B-e). The teratoma formation rate was 100% (2/2). In EBs at days 7, 14, 21, and 28 of culture, we examined the expression of the germ cell markers *CD9*, *DPPA3* (*STELLA*), and *DDX4* (*VASA*) by RT-PCR. Expression of *CD9* and *DPPA3* was detected in all samples, while *DDX4* was not, although two primer sets for *DDX4* were used (Fig. 3C).

Characterization of single-cell subcultured ES cells

To further characterize the AgMES cell line, we examined whether this cell line could be subcultured by dividing it into single cells with trypsin. The average replating efficiency and rate of colonies with undifferentiated morphology until 52 passages were 20.2±5.7

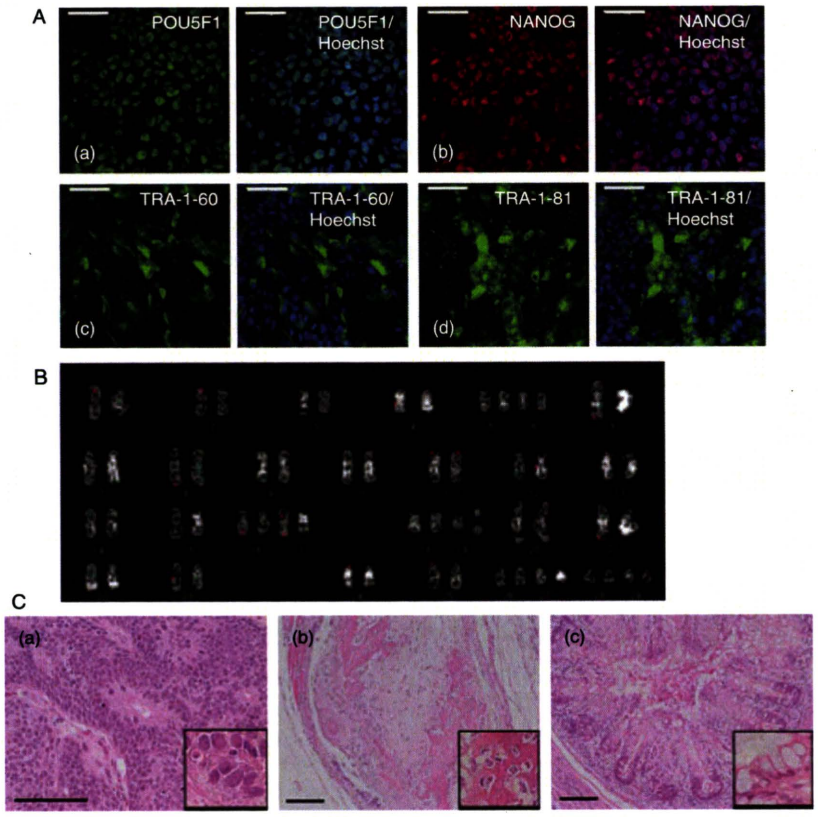

Figure 4 Characterization of single-cell subcultured AgMES cells. (A) The expression of the pluripotency markers, POU5F1 (a), NANOG (b), TRA-1-60 (c), and TRA-1-81 (d), was confirmed. Nuclei were counterstained with Hoechst 33342 (blue) (a–d). A double staining with POU5F1 (a) and NANOG (b) was conducted in the same specimen. Bar represents 50 μm. (B) Karyotyping of single-cell subcultured AgMES cells. At passage 34, 80% (40/50) of the single-cell subcultured AgMES cells had a normal karyotype (60 chromosomes, XX). (C) Histological analysis of teratomas formed from single-cell subcultured AgMES cells. The teratomas showed neural (ectoderm, a), osseous (mesoderm, b), and gut (endoderm, c) tissues. Each figure includes insets for showing high magnification of typical cells, such as neuroblasts (a), osteoblasts with osseous matrix (b), and goblet cells (c). Bars represent 100 μm, 40 μm (inset a), and 20 μm (inset b and c).

and 15.4±5.8% respectively and 74.5±13.8% of the subcultured single cells formed the undifferentiated colonies. Immunofluorescence revealed that this cell line strongly expressed POU5F1, TRA-1-60, TRA-1-81, and NANOG but not SSEA1 (Fig. 4A). Karyotyping analysis revealed that 80% (40/50) of cells examined at passage 34 after single-cell subculture had a normal chromosome number of 60 (Fig. 4B). Histological analysis revealed that teratomas by single-cell subcultured ES cells in two immunodeficient mice consisted of three embryonic germ layer tissues, e.g. neural and dermal tissues as ectoderm (Fig. 4C-a; neural tissue), musculoskeletal, fatty, fibrous, and vascular tissues as mesoderm (Fig. 4C-b; osseous tissue), and gut tissue as endoderm tissue (Fig. 4C-c). The teratoma formation rate was 100% (2/2).

Discussion

We examined *in vitro* culturing of ICSI-derived zygotes and the establishment of an ES cell line, rather than examining developmental potential to term, in the African green monkey. As a result, we succeeded in developing the zygotes to the blastocyst stage and were able to establish a novel primate ES cell line from nine of the blastocysts. The cells derived from African green monkeys are utilized in many types of research and tests. Therefore, it is expected that the AgMES cell line described in this report could contribute to these applications as a novel biological resource.

Reproductive technologies in the African green monkey have hardly been reported. Development to the blastocyst stage was only reported by *in vitro* culture in a simple medium following production of the fertilized eggs with IVF in 1997 (Sankai *et al.* 1997). We reported follicle growth stimulus methods and the fertilization ability by ICSI in 2007 (Shimozawa *et al.* 2007). Herein, we examined an *in vitro* culture system using zygotes produced by ICSI. *In vitro* culturing with and without a MEF cell monolayer supported development to the blastocyst stage, while culturing with a BRL cell monolayer did not. Although a BRL cell monolayer supported development to the blastocyst stage of rhesus monkey embryos (Zhang *et al.* 1994, Nusser *et al.* 2001), the reason for this negative effect by BRL cells in African green monkey embryos in the present study is uncertain. On the other hand, the ES cell line was established from the blastocysts that originated from the culture system with the MEF cell monolayer. The number of cells constituting the blastocysts or their ICMs could not be examined because we gave priority to the establishment of the ES cell line. Consequently, it was impossible to compare effects between the culture systems in detail. However, one of the reasons for the successful

establishment of an ES cell line may have been that MEF cells greatly affected the quality of the ICM.

The novel established AgMES cell line showed characteristics similar to those of other primate ES cell lines (Thomson *et al.* 1995, 1996, 1998, Suemori *et al.* 2001). The ratio of nuclei to the cytoplasm in an ES cell was very high, and the colony morphology was flat. Expression of the primate ES cell-specific markers was confirmed by immunofluorescence staining and RT-PCR analysis, and we confirmed that ES cells spontaneously differentiated into three embryonic germ cells via the EBs under *in vitro* culturing and formed teratomas that consisted of three embryonic germ cells by being transplanted to SCID mice. In addition, 88% of the examined cells had maintained the normal chromosome number of 60. As described above, the cell line established herein demonstrated that it is an exact match to the ES cell line.

Immunofluoresence revealed that AgMES vaguely expressed SSEA3 and SSEA4. This may be due to antibodies not suitable for African green monkey epitopes. However, it is possible that expressions of SSEA3 and SSEA4 are not indispensable for ES cells (Brimble *et al.* 2007). The cynomolgus monkey ES cells are used for basic experiments aimed at application in regenerative medicine (Sánchez-Pernaute *et al.* 2005, Takagi *et al.* 2005, Shibata *et al.* 2006, Osakada *et al.* 2008). Reports of the differentiation from cynomolgus monkey ES cells to various types of cell suggest that the expression of SSEA3 may not be necessary.

The gene expression analysis also showed that AgMES cells were positive for *POU5F1*, *NANOG*, *SOX2*, and *REX1*. The expression of *FOXD3* was not detected, despite the use of three primer sets for *FOXD3*. However, in EB, the expression of *FOXD3* was detected (data not shown). It has been reported that *FOXD3* has the antagonistic effect on *POU5F1* (Guo *et al.* 2002) and was not expressed in undifferentiated human and common marmoset ES cells (Ginis *et al.* 2004, Mandal *et al.* 2006, Müller *et al.* 2009). We demonstrated that *FOXD3* of African green monkey was not expressed in undifferentiated cells but was detected during differentiation the same as human and common marmoset. In addition, a difference in the gene expression of the pluripotency marker *REX1* in ES cells has been observed among species. *REX1* expression has been detected in human ES cells (Ginis *et al.* 2004, Mandal *et al.* 2006) but not in rhesus monkey ES cells (Mitalipov *et al.* 2006). In the present study, both of African green and cynomolgus monkey ES cells expressed *REX1*, suggesting that the characterization of different ES cell lines within the same species may differ.

Several researchers have examined the differentiation of primate ES cells into germ cells (Clark *et al.* 2004, Chen *et al.* 2007, Sparman *et al.* 2009). When we examined the expression of three germ cell markers, *CD9*, *DPPA3*, and *DDX4*, in EBs derived from AgMES cells by RT-PCR, the expression of *CD9* and *DPPA3* was detected. This suggested that AgMES cells might have the potential ability to differentiate into germ cells, although further study is necessary. Differentiation into germ cells may be enabled in the primates by doing the comparative study using primate and mouse ES cell lines. Additionally, it was reported that epiblast stem cells (EpiSCs) established in mice did not possess the ability to differentiate into germ cells *in vivo* (Brons *et al.* 2007, Tesar *et al.* 2007). Primate ES cells may also lack the potential to differentiate into germ cells because EpiSCs show characteristics similar to primate ES cells such as culture condition and colony morphology. However, it is unclear whether primate ES cells can differentiate into germ cells. It will thus be very important to study the differentiation of nonhuman primate ES cells into germ cells both *in vivo* and *in vitro*. We consider that the AgMES cells could become a highly valuable research tool as well as rhesus, cynomolgus, and common marmoset monkey ES cell lines.

Next, we examined whether single AgMES cells can continuously be passaged. When the subcultures of single ES cells dissolved with trypsin were repeated 52 times, a replating rate of 20.2% and an undifferentiation colony formation rate of 15.4% were obtained. These ES cells were positive for the primate ES cell-specific markers, formed teratomas, and had normal karyotype, which demonstrated that the AgMES cells maintained by the single-cell subculture method had the same characteristics of undifferentiation and pluripotency. Human ES cell lines were difficult to continue to subculture by single cells, and because the undifferentiation colony formation rate was ~1%. Improvements of culture environment by using some feeder cells or chemical compounds have been examined (Amit *et al.* 2000, Hasegawa *et al.* 2006, Ellerström *et al.* 2007, Watanabe *et al.* 2007). However, single AgMES cells showed 15% of the undifferentiation colony formation rate in the same culture environment as the subculture by small clusters. The AgMES cell line has the potential for easy application in various examinations under *in vitro* culture and to contribute to the research into the proliferation mechanisms underlying colony formation from a single cell in the primate ES cells.

The primary culture cells or the Vero cells that originate from the kidney of the African green monkey have been used for applications such as vaccine developments and virus inspections. In addition, the red blood cells of the African green monkey are used for the measles virus test. We think that studies of the erythropoiesis or hematopoiesis differentiation in cynomolgus ES cells might be able to contribute to differentiation of the red blood cells from AgMES (Hiroyama *et al.* 2006, Umeda *et al.* 2006). The characteristics and chromosomes of kidney-derived cells may have mutated naturally by repeating the subculture for a long time. Inducing differentiation of

pluripotent ES cells, whose chromosomes are relatively steady compared with the various cell lines, may facilitate their becoming a source of cells with uniform properties. This shows the possibility that the red blood cells and the cells of kidney differentiated from the AgMES cells may become a substitute for cells from living monkeys. Producing the various cells from the ES cells would alleviate the need to collect the cells from living animals.

Materials and Methods

Animals

The mature African green monkeys (*C. aethiops*) used in this study were bred and maintained in an air-conditioned room with controlled illumination (12 h light:12 h darkness), temperature (25±2 °C), humidity (60±5%), and ventilation (10 cycles/h), and were given 70 g of commercial food (Type AS; Oriental Yeast Co., Ltd, Tokyo, Japan) and 100 g of apples daily, and unlimited access to tap water at the Tsukuba Primate Research Center (Tsuchida *et al.* 2008). Every morning, the health condition (e.g. viability, appetite, fur-coat appearance) and menstruation status of each female monkey were monitored. For ovarian stimulation, eight female monkeys were used. Maintenance of animals was conducted according to the guidelines set by the National Institutes of Biomedical Innovation (NIBIO) for the care, use, and biohazard countermeasures of laboratory animals. This study was approved by the Animal Welfare and Animal Care Committee of NIBIO.

Ovarian stimulation and oocyte collection

For ovarian stimulation, the administration of human FSH (hFSH: Fertinorm, Merck Serono), equine chorionic gonadotropin (eCG: Serotropin, ASKA Pharmaceutical, Tokyo, Japan), or human menopausal gonadotropin (hMG) followed by human chorionic gonadotropin (hCG: Gonatropin, ASKA Pharmaceutical) was conducted. Protocols of hFSH and eCG administration, and oocyte collection were reported previously (Shimozawa *et al.* 2007). Briefly, on the first day of menstruation, the female monkeys were administered leuprorelin acetate s.c. as a GNRH agonist (GNRHa; Leuplin, Takeda Pharmaceutical, Osaka, Japan). At 2–3 weeks after that, the follicular growth of these monkeys was stimulated by 25 IU/kg per day hFSH administered for 9 days, 200 IU/head per day eCG administered i.m. for 6 days, or 37.5 IU/head per day hMG administered i.m. daily for 6 days. Thirty-six hours after the final hFSH, eCG, or hMG administration, 1200 IU/head hCG was administered. At 36–38 h after hCG administration, females were anesthetized by 10 mg/kg ketamine hydrochloride (Ketalar, Bayer Yakuhin) and 1 mg/kg xylazine hydrochloride (Seractarl, Bayer Yakuhin) administration. Ovaries were exposed through an abdominal incision, and the contents of the follicles were aspirated through a 25-gauge needle. The collected follicular contents were immediately placed in TYH medium modified by adding Hepes (Hepes-TYH) containing 2.5 IU/ml heparin (Novo Nordisk Pharma, Tokyo, Japan). The oocytes were freed from cumulus cells by pipetting after treatment with 0.1% hyaluronidase (Sigma) in Hepes-TYH medium. Collected oocytes were then transferred to CMRL-1066 (Invitrogen) medium containing 10% fetal bovine serum (FBS, Hyclone, Logan, UT, USA), Gluta-MAX (×100, Invitrogen), and penicillin–streptomycin solution (×100, Sigma), hereafter simply called CMRL.

ICSI and embryo culture

Sperm collection and ICSI were performed as described previously (Shimozawa *et al.* 2007). Briefly, fresh semen was collected in TYH medium by rectal probe electrostimulation. Semen suspension was layered onto 90% Percoll (GE Healthcare UK Ltd, Buckinghamshire, England) diluted with 9% NaCl solution and then centrifuged at 800 ***g*** for 10 min. The precipitate containing sperm was suspended in CZB modified by adding Hepes (Hepes-CZB). Sperm suspended in Hepes-CZB with 10% polyvinylpyrrolidone (Sigma) was used for ICSI. Mature oocytes with a polar body were subjected to ICSI using a micromanipulation system equipped with a piezo drive unit (Primetech, Ibaraki, Japan) under an inverted microscope (Nikon, Tokyo, Japan). An immobilized spermatozoon drawn into the injection pipette was injected into the mature (MII) oocyte by using a few piezo pulses, and then injected oocytes were transferred into CMRL microdroplets. The fertilized embryos were judged to be normal based on the formation of two pronuclei and the release of a second polar body 15–16 h after ICSI. Embryos were cultured with or without a MEF cell monolayer or a BRL cell monolayer in 500 μl CMRL using a four-well multidish (Nunc, Rochester, Denmark). Half of the CMRL was changed every other day. Culturing was performed under CMRL covered with mineral oil at 37.5 °C in a humidified atmosphere of 5% CO_2, 5% O_2, and 90% air.

Establishment of ES cells

The TE and ZP of blastocysts were removed mechanically with 27-G needles or dissolved in the ZP with 0.5% actinase. Treated blastocysts were cultured with mitomycin C-treated STO cell or MEF cell monolayer on gelatin-coated dishes in ESM consisting of DMEM/F12 (1:1) supplemented with 20% knockout serum replacement (KSR, Invitrogen), 1% GlutaMax (Invitrogen), 0.1 mM β-mercaptethanol (Sigma), 1% nonessential amino acids (Invitrogen), and 10 ng/ml human recombinant leukemia inhibitory factor (hLIF, Millipore, Billerica, MA, USA). In one case, ESM in which FBS replaced KSR and that was supplemented with 4 ng/ml human recombinant basic fibroblast growth factor (hbFGF, Wako Pure Chemical Industries, Ltd, Osaka, Japan) was used. The extended colony was treated with 0.1% collagenase (Wako) in DMEM (Sigma) and divided mechanically into small clusters with pipetting or with 27-G needles, then passaged onto a new feeder layer. The resulting stem cell colony was expanded using 0.1% collagenase and passaged with pipetting or 27-G needles into a 6-cm dish (BD Falcon, Franklin Lakes, NJ, USA). ES cell colonies were divided into small clusters using 0.1% collagenase and pipetting.

Table 2 Primer sequences used for RT-PCR analyses.

Gene	Forward	Reverse	bp
POU5F1	TTGGAGACCTCTCAGCCTGA	ACACATGTTCTTGAAGCTAA	326
SOX2	CCCCCGGCGGCAAC GCA	TCGGCGCCGGGGAGATACAT	448
NANOG	CAGAAGGCCTCAGCACCTAC	GACTGTTCCAGGCCTGATTGTT	217
REX1	CAGATCGAAAACAGCTCGCAGAAT	CGTACGCAAATTGAAGTCCAGG	305
FOXD3	TACATCGCGCTCATCACCATG	GTTGTCGAACATGTCCTCGGA	246
CD9	AAATAGCTGCGGCCATCTGGGGATA	G CCCCCAGCCAAACCACAGCA	167
DPPA3	GTTACTCGGCAGAGTTCGTA	TGAAGTGGCTTGGTGTCGTA	167
DDX4	AGGATGAGGACTCCATCTTTGCACATTAT	CAGACCCTGTTTGAGCACAAGCCA	252
GAPDH	TGGACCTGACCTGCCGTCT	GGAAGAGTGGGTGTCGCTGT	152

For the investigation of undifferentiated colony formation from a single ES cell, ES cell colonies recovered after collagenase treatment were dispersed to single cells with 0.25% trypsin/0.1 m EDTA (Sigma), and a thousand single ES cells were cultured with MEF cell monolayer in a 3.5-cm dish (BD Falcon).

Characterization

Alkaline phosphatase was detected using Alkaline Phosphatase Chromogen Kit (Biomeda, Plovdiv, Bulgaria). For immunofluorescence, cells were fixed with 4% paraformaldehyde in PBS for 20 min. Following permeabilization with 0.2% Triton X-100 in PBS for 10 min and blocking with 5% skim milk in PBS for 30 min, cells were incubated with primary antibodies overnight at 4 °C and visualized by IgG or IgM conjugated with Alexa 488 (A11001, A21042, and A21212) or 555 (A21428) (1:1000, Invitrogen). Primary antibodies used were as follows: POU5F1 (1:50, 611202; BD Biosciences, San Jose, CA, USA), NANOG (1:50, RCAB0003P, ReproCELL Inc., Tokyo, Japan), SSEA1 (MAB4301), SSEA3 (MAB4303), SSEA4 (MAB4304), TRA-1-60 (MAB4360), TRA-1-81 (MAB4381) (1:80, Millipore), Nestin (1:50, MAB1259), α-smooth muscle actin (1:100, MAB1420, R&D Systems, Minneapolis, MN, USA), β-tubulin III (1:50, T 8660), α-fetoprotein (1:500, A 8452, Sigma), and CDX2 (1:100, GTX15258, GeneTex, San Antonio, TX, USA). The nuclei were stained with 10 μg/ml Hoechst 33342 (Calbiochem, Darmstadt, Germany) in PBS.

For pluripotency analysis, EBs and teratomas were prepared from ES cells. EBs were grown by culturing unattached ES cell colonies in ESM without hLIF and hbFGF. After 2 weeks, EBs were transferred to 6-cm dishes in DMEM containing 10% FBS for attachment culture. Spontaneous differentiated cells from EB were observed and analyzed by immunofluorescence. Teratoma formation was as follows: six 6-cm dishes of small cluster subcultured and single-cell subcultured ES cells suspended in ESM without hLIF and hbFGF were injected into the hind leg muscle of two immunodeficient mice (NOD/SCID, Charles River Japan, Kanagawa, Japan) respectively. Tumors were fixed with 4% paraformaldehyde in PBS, then embedded in paraffin, and sectioned for histological analysis by hematoxylin and eosin staining. No tumors formed in two NOD/SCID mice that were injected with ESM without hLIF and hbFGF as a control.

Karyotype analyses were performed at the International Council for Laboratory Animal Science Monitoring Center (Kanagawa, Japan) or the Chromosome Science Labo Inc (Hokkaido, Japan).

Gene expression analysis

Undifferentiated AgMES cells and EBs at days 7, 14, 21, and 28 of culture were treated with RNA*later* (Ambion, Austin, TX, USA). RNA was isolated using an RNAqueous Kit (Ambion) according to the manufacture's protocol. First-strand cDNA was primed via random hexamers, and RT-PCR was performed with the primer sets showing in Table 2. The expected sizes of the PCR products were inferred from rhesus monkey and human sequences.

Statistical analysis

Data were analyzed using Fisher's exact probability test. $P<0.05$ was considered significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Intradermal Delivery of Recombinant Vaccinia Virus Vector DIs Induces Gut-Mucosal Immunity

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Abstract

Antigen-specific mucosal immunity is generally induced by the stimulation of inductive mucosal sites. In this study, we found that the replication-deficient vaccinia virus vector, DIs, generates antigen-specific mucosal immunity and systemic responses. Following intradermal injection of recombinant DIs expressing simian immunodeficiency virus *gag* (rDIsSIV*gag*), we observed increased levels of SIV p27-specific IgA and IgG antibodies in faecal extracts and plasma samples, and antibody-forming cells in the intestinal mucosa and spleen of C57BL/6 mice. Antibodies against p27 were not detected in nasal washes, saliva, and vaginal washes. The enhanced mucosal and systemic immunity persisted for 1 year of observation. Induction of Gag-specific IFN-γ spot-forming $CD8^+$ T cells in the spleen, small intestinal intraepithelial lymphocytes, and submandibular lymph nodes was observed in the intradermally injected mice. Heat-inactivated rDIsSIV*gag* rarely induced antigen-specific humoral and T-helper immunity. Moreover, rDIsSIV*gag* was detected in MHC class II IA antigen-positive (IA^+) cells at the injection site. Consequently, intradermal delivery of rDIs effectively induces antigen-specific humoral and cellular immunity in gut-mucosal tissues of mice. Our data suggest that intradermal injection of an rDIs vaccine may be useful against mucosally transmitted pathogens.

Introduction

As most infectious agents, including human immunodeficiency virus (HIV), are often transmitted via mucosal surfaces, a mucosal-inductive vaccine capable of eliciting protective immunity in mucosal tissues and external secretions would act as the first line of defence at the site of initial invasion. For inducting preventive immunity to HIV, a vaccine must induce anti-HIV neutralizing antibodies (Ab) and/or cytotoxic T lymphocytes against HIV-infected cells in the mucosal and submucosal areas [1]. Parenterally immunized vaccines generally do not enhance the levels of secretory IgA Ab production in external secretions and are less able to induce the mucosal immune responses needed to prevent infection at the site of initial contact between the host and the infectious agent [2–4].

Poxvirus vectors are among the most heavily exploited for vaccine development. Their use is largely attributable to the overwhelming success of the vaccinia virus vaccine in eradicating smallpox. Because of concerns regarding the use of a replicating vector in immunocompromised individuals, non-replicating poxvirus vectors, such as the modified vaccinia virus Ankara (MVA) [5], are an area of interest for extensive development. The replication-deficient vaccinia virus, DIs, is a candidate viral vector; and it is a safe and highly attenuated mutant of the vaccinia virus [6–9]. When recombinant DIs (rDIs) encoding foreign antigens (Ag) was intravenously [9, 10], intramuscularly [11, 12], subcutaneously [13], or intradermally [14] injected, Ag-specific systemic immunity was induced in mice and non-human primates.

Activation of the inductive sites is of paramount importance to induce Ag-specific mucosal immunity. Intradermal and intramuscular injection of a DNA vaccine generates solely systemic but rarely mucosal responses [15, 16]. The potential importance of specific mucosal immunity in the protection against mucosally transmitted pathogens is beginning to emerge from many investigations. We had previously demonstrated that intranasal or intragastrical administration of rDIs encoding full-length simian immunodeficiency virus *gag*

(rDIsSIV*gag*) could induce Gag-specific cytotoxic T lymphocytes and p27-specific Ab in both the systemic sites and the mucosal sites of mice [17].

In recent clinical studies, the smallpox vaccine was administered by skin scarification, subcutaneous, intramuscular, or intradermal vaccination [18–20]. Immunization of the mice with MVA by skin scarification protected the mice against intranasal challenge with pathogenic Western Reserve vaccinia virus in mice, but intramuscular immunization was ineffective [21]. Subcutaneous injection of rDIs was inefficient to induce Ag-specific mucosal IgA Ab responses whereas high IgG Ab responses were induced in the plasma [13]. DIs vaccines are typically parenterally injected; however, mucosal immunity has not been tested in parenterally injected animals. An advantage of intradermal injection is that all the materials for intradermal injection are readily available and accessible to clinicians. Therefore, we studied whether intradermal injection of rDIs induces mucosal immunity. Here, we describe the enhanced induction of humoral and cellular immunity in the mucosal tissues of mice injected intradermally with the DIs vector.

Materials and methods

Recombinant vaccinia virus vectors. The production and preparation of rDIsSIV*gag* and rDIs expressing LacZ (rDIsLacZ) as a control vector have been described in detail previously [9, 11]. To prepare heat-inactivated rDIsSIV*gag*, 10^5 PFU of rDIsSIV*gag* was incubated at 56 °C for 30 min. The PFU of heat-inactivated rDIsSIV*gag* was confirmed as < 2 by a chicken embryo fibroblast culture [22, 23]. These vectors were stored at −80 °C until used.

Mice. Five-week-old C57BL/6N mice were purchased from Charles River Japan, Inc. (Yokohama, Japan). The mice were acclimated to the experimental animal facility for more than 1 week before being used in the experiments. They were maintained in the facility under pathogen-free conditions. All experimental procedures were performed in accordance with the guidelines established by the National Institute of Infectious Diseases, Japan. The study was conducted in a biosafety level 2 facility under the approval of an institutional committee for biosafety and in accordance with the requirements of the World Health Organization. In cases of specification, systemic vaccination against pathogenic viruses or bacteria induced mucosal secretory IgA Ab responses in individuals who had previously been exposed to its pathogen by the mucosal route [24–26]. That, of course, is negated here because we used naive mice in this study, and p27-specific Ab were not detected in the plasma and faecal extracts of the mice before immunization.

Immunization. The mice were injected intradermally at the interscapular region thrice at weekly intervals in two contiguous sites with a 25-μl aliquot of PBS containing 10^5 PFU per 50 μl of rDIsSIV*gag* or rDIsLacZ. The mice were extensively washed with warm water, blot-dried, and then washed and dried again to avoid acquiring small amounts of injected rDIs through their normal grooming activity. To compare with the mucosal immunization, the mice were immunized with 10^5 PFU of rDIsSIV*gag* by the nasal route thrice at weekly intervals according to previously described methods [17].

Sample collection and preparation of single-cell suspensions. After immunization, blood and mucosal secretions (faecal extracts, nasal washes, saliva, and vaginal washes) were collected by using methods described elsewhere [27–30]. Vaginal washes were pooled from four mice for each experiment [30]. The collected samples were stored at −80 °C until used. Single-cell suspensions were obtained from the spleen, mesenteric lymph nodes (MLN), submandibular lymph nodes (SMLN), axillary lymph nodes (ALN), small intestinal lamina propria (i-LP), and small intestinal intraepithelial lymphocytes (IEL) as previously described [29].

Analysis of IFN-γ production of SIV Gag peptide-specific $CD8^+$ T cells. To detect Gag-specific cellular immunity, $CD8^+$ T cells in the spleen, IEL, and SMLN of mice were cultured with or without overlapping Gag peptide. The methods of $CD8^+$ T cell enrichment and Gag-specific IFN-γ spot-forming cells (SFC) assessed by enzyme-linked immunosorbent spot (ELISPOT) assay were as described previously [17]. The number of Gag-specific IFN-γ SFC was calculated by subtracting the results of the control culture (i.e. without Gag peptide stimulation) from those of the peptide-stimulated culture, because non-specific activated $CD8^+$ T cells produced IFN-γ.

Detection of SIV p27-specific Ab production by ELISA and enumeration of p27-specific Ab-forming cells by ELISPOT assay. Titres of p27-specific Ab in the plasma and mucosal secretions were determined by an endpoint enzyme-linked immunosorbent assay (ELISA). The endpoint titres were expressed as the last dilution that gave an optical density at 450 nm (OD_{450}) of ≥ 0.1 OD units above the OD_{450} of the negative controls [17]. SIV p27-specific IgA, IgG, and IgM Ab-forming cells (AFC) in the spleen, i-LP, and MLN of mice were determined by ELISPOT assay. SIV p27-specific AFC were quantitated with the aid of a stereomicroscope [17].

Cytokine production of SIV Gag peptide-specific $CD4^+$ T cells. Overlapping Gag peptide-specific helper immunity of $CD4^+$ T cells in the spleen of mice was measured by ELISA. The methods of $CD4^+$ T-cell purification and culture conditions were as described previously [17]. The levels of IFN-γ, IL-4, and IL-10 were measured by ELISA kit (eBioscience, San Diego, CA, USA). The levels of Gag-specific IFN-γ, IL-4, and IL-10 in the culture supernatants were calculated by subtracting the results of the

control cultures from those of the Gag peptide-stimulated cultures.

Isolation of IA$^+$ cells in the skin. Twenty-four hours after the intradermal injection of 10^7 PFU rDIsSIV*gag*, the mice were shaved at the vaccinal locus with a razor. The skin was then removed and cut to approximately 1×1 cm^2 of the vaccinal locus. MHC class II IA antigen-positive (IA$^+$) cells were isolated from the skin by enzymatic dissociation protocols [31]. The epidermal cells were incubated with anti-I-A^b monoclonal Ab (AF6-120.1; Becton Dickinson, San José, CA, USA). IA$^+$ cells were isolated by auto MACS (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). The purified fractions included > 97% IA$^+$ cells.

Preparation of DNA samples and amplification of SIV gag gene by nested PCR. For determining the distribution of rDIsSIV*gag*, a nested PCR was used to amplify a fragment of the *gag* gene segment. DNA samples were prepared from the skin, skin IA$^+$ cells, ALN, SMLN, Peyer's patches (PP) and spleen. The preparation, amplification methods, and primer sequences are described elsewhere [17, 32]. The lowest concentration of plasmid SIV DNA detectable with this PCR method in the first amplification with an outer *gag* primer pair was 10^3 copies. Upon further amplification with nested/internal *gag* primers, a single copy of plasmid DNA could be routinely detected [32].

Statistical analysis. Normally distributed variables were compared by the two-tailed Student's *t*-test, and the results are expressed as the mean ± the standard deviation (SD). Non-normally distributed variables were compared by the two-tailed Mann–Whitney *U* test, and the results are expressed as the median and the interquartile range (IQR). A P value < 0.05 was considered significant.

Results

Cellular and humoral immunity by intradermal injection of rDIs

The first step was to assess whether intradermal injection of rDIsSIV*gag* induces Gag-specific IFN-γ-producing CD8$^+$ T cells in mucosal and systemic tissues. Although the BCG vector itself non-specifically enhances the levels of some cytokines [33], the DIs empty vector and rDIs-LacZ scarcely stimulated IFN-γ-production, and the number of IFN-γ SFC per 10^6 cells was less than 32 in intradermally injected mice [14] and intramuscularly injected non-human primates [12] in our previous studies. Moreover, the calculated number of Gag-specific IFN-γ SFC from naive mice was < 15 per 10^6 CD8$^+$ T cells. A calculated number of SFC above 15 was considered positive. The number of Gag-specific IFN-γ SFC in the spleen in the intradermal injection group was significantly higher than that of the mice immunized intranasally with the same dose of rDIsSIV*gag*. Conversely, the number of IFN-γ SFC in the IEL of the mice injected intradermally was significantly lower than that of the intranasally immunized mice. There was no significant difference in the SMLN between the intradermally injected group and the intranasally immunized group (Fig. 1). Gag-specific IFN-γ-producing CD8$^+$ T cells were apparent in both mucosal and systemic tissues in the intradermally injected mice.

Ag-specific Ab, especially secretory IgA Ab in mucosal secretions, have been shown to be of central importance for host defence [1, 34]. We assessed the Ab response to p27 in the plasma and faecal extracts of immunized mice at 1 week after the last immunization. Intradermal injection of rDIsSIV*gag* induced p27-specific IgG Ab in the plasma, and p27-specific IgA Ab in the faecal extracts in six of the eight (75%) mice at 1 week after the last intradermal injection. Remarkably, the titres of p27-specific IgA Ab in the faecal extracts of mice immunized intradermally or intranasally with rDIsSIV*gag* were roughly equivalent (Fig. 2). However, p27-specific IgA Ab were not detected in the nasal washes, saliva, and vaginal washes of intradermally injected mice (data not shown). The p27-specific IgG and IgA Ab titres in the plasma and faecal extracts from the mice intradermally injected with rDIsLacZ (negative controls) were less than the detection limit on endpoint ELISA among (data not shown).

Intragastric immunization with rDIsSIV*gag* has been reported to induce p27-specific IgA Ab in the faecal

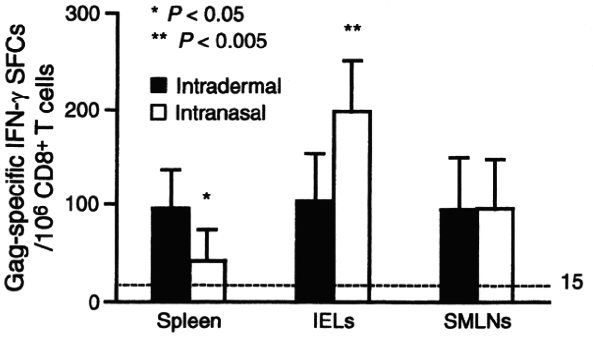

Figure 1 Intradermal injection of rDIsSIV*gag* induced SIV Gag-specific IFN-γ-secreted CD8$^+$ T cells in both mucosal tissues and systemic tissue. CD8$^+$ T cells were isolated one week after the last injection from the spleen, IEL, and SMLN of the mice injected intradermally (closed column) or intranasally (open column) with 10^5 PFU of rDIsSIV*gag*. 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 per 10^6 CD8$^+$ T cells + SD for eight mice in each group and are representative of two separate experiments. Each group was compared by two-tailed Student's *t*-test. Significant differences between the intranasal group and the intradermal group are indicated with an asterisk (*P < 0.05, **P < 0.005).

