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.

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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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

Infant ID	Method of delivery	Dam ID	Method of nursing	Status of dam at parturition		Status of infant at				
						0 d	1 mo	2 mo	Weaning (approximately 6 mo)	
				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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SHORT PAPER

Acute Megakaryocytic Leukaemia (AMKL)-like Disease in a Cynomolgus Monkey (*Macaca fascicularis*)

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Summary

A 5-year-old male cynomolgus monkey (*Macaca fascicularis*) with a clinical history of bleeding tendency, severe anaemia, thrombocytopenia and elevated serum concentration of liver-related enzymes was examined *post mortem*. Ecchymotic haemorrhages were present on the left eyelid and forehead. The liver, kidney and spleen were markedly enlarged and the kidneys had capsular petechiae. Microscopically, numerous atypical cells resembling myeloid cells were observed in the bone marrow, and myelofibrosis was present. Atypical cells were also present in the blood vessels of the liver, kidney, spleen, lymph nodes, lung, heart, bladder, adrenal gland and brain. Some neoplastic cells had oval or pleomorphic macronuclei and others were multinucleated. Immunohistochemically, the majority of the neoplastic cells had granular cytoplasmic expression of the megakaryocyte-associated antigens Von Willebrand Factor and CD61-IIIa, but were negative for myeloperoxidase. A diagnosis of acute megakaryocytic leukaemia (AMKL)-like disease was made. This would appear to be the first report of AMKL-like disease in non-human primates. This monkey was infected with simian retrovirus type D and it is possible that this viral infection was associated with the development of neoplasia.

Keywords: acute megakaryocytic leukaemia; cynomolgus monkey; immunohistochemistry; simian retrovirus type D

Haematological malignancy has been infrequently documented in monkeys infected by the simian immunodeficiency virus (SIV) (Fortgang *et al.*, 2000). Simian T-cell leukaemia virus (STLV) is also linked to the development of simian T-cell malignancies that closely resemble human T-lymphotropic virus (HTLV) associated leukaemia and lymphoma (Hubbard *et al.*, 1993). Furthermore, simian retrovirus type D (SRV/D) is a common cause of simian acquired immunodeficiency syndrome (SAIDS), a fatal immunosuppressive disease of macaques. SRV/D-infected monkeys may develop lymphadenopathy, splenomegaly, anaemia, bone marrow hyperplasia, lymphoid depletion, neutropenia, weight loss, diarrhoea or malignant neoplasia (Guzman *et al.*, 1999). Although a number of clinical and pathological studies have described lymphoma in non-human primates (Hubbard *et al.*, 1993; Paramastri *et al.*, 2002), there are no reports of myeloid leukaemia in these animals. The present report describes the first case of acute megakaryocytic leukaemia (AMKL)-like disease in a non-human primate.

A 5-year-old male cynomolgus monkey (*Macaca fascicularis*) was housed in the Tsukuba Primate Research Center (TPRC) in an individual cage and maintained according to the National Institute of Biomedical Innovation rules and guidelines for experimental animal welfare. On routine haematological examination, the animal was found to have mild anaemia (red blood cells [RBC] 4.28×10^{12}/l; reference range $5.55–6.63 \times 10^{12}$/l; haemoglobin [Hb] 99 g/l; reference range 105–125 g/l; haematocrit [HCT] 32.7%; reference range 35.4–41.4%) and severe thrombocytopenia (platelets [PLT] 27×10^9/l; reference range $195–339 \times 10^9$/l). The number of white blood cells (WBC) was normal (6.9×10^9/l; reference

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range 4.2–9.2 × 10^9/l). Although the animal care staff regularly monitored the health of the animal, at this time no clinical signs were observed. Repeat haematological examinations were performed one and four weeks later, but there was no progression of the anaemia and thrombocytopenia was not present on these occasions. The monkey continued to have normal appetite, faeces and activity.

Three months after the initial haematological examination a spot of blood was detected under the monkey's cage. At this time the animal displayed clinical signs including emaciation, pallor of mucous membranes and haemorrhage on the cutaneous side of one eyelid. Haematological examination revealed severe anaemia (RBC 1.66 × 10^{12}/l, Hb 39 g/l, HCT 13.3%) and thrombocytopenia (PLT 28 × 10^9/l). The WBC count was normal (4.1 × 10^9/l). Serum biochemical examination revealed elevation in the concentration of aspartate aminotransferase (AST, 176 U/l; reference range 31–47 U/l); alanine aminotransferase (ALT, 303 U/l; reference range 21–65 U/l); lactate dehydrogenase (LDH, 7660 U/l; reference range 292–975 U/l), and C reactive protein (CRP, 12.8 mg/l; reference range 0.3–1.7 mg/l).

Cynomolgus monkeys in the TPRC breeding colony are SIV and STLV negative, but most are infected by SRV/D (Hara *et al.*, 2005). The animal described here was seronegative for SRV/D antibody by western blotting, but tested positive by polymerase chain reaction (PCR) for the detection of virus genetic material, consistent with current viraemia. On the basis of the clinical and laboratory data, haematological malignancy was suspected.

The monkey was deeply anaesthetized with a lethal dose of pentobarbital and necropsy examination was performed. Ecchymoses were noted on the left eyelid and forehead. The liver was markedly enlarged and the gallbladder was distended. The kidneys were enlarged, pale red-brown in colour and had capsular petechiation. The spleen was also enlarged, but there were no distinct lymphoid follicles on the cut surface. A dark red nodule (1 cm diameter) was present within each of the inferior lobes of the lung. The femoral bone marrow had a brownish-red appearance.

Tissues were fixed in 10% neutral buffered formalin, processed routinely and embedded in paraffin wax. Sections (3 μm) were stained with haematoxylin and eosin (HE), periodic acid-Schiff (PAS) and Masson's trichrome stains. Microscopically, many atypical cells resembling myeloid cells were observed in the bone marrow and the blood vessels of the liver, kidney, spleen, lymph nodes, lung, heart, bladder, adrenal gland and brain. Some hepatic and renal vessels contained neoplastic emboli (Fig. 1). There was extensive infiltration of the liver, kidneys and adrenal

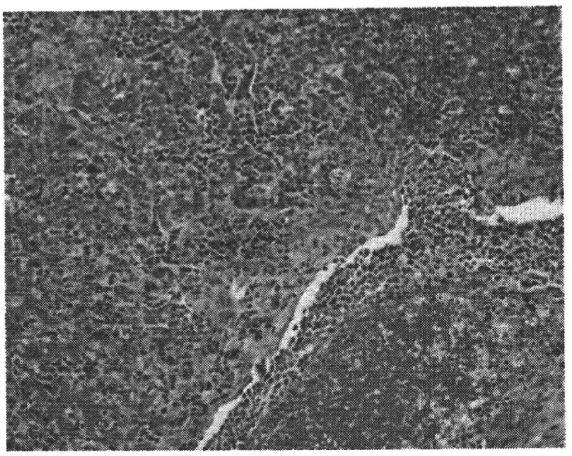

Fig. 1. Extensive infiltration of neoplastic cells into the hepatic parenchyma with associated degeneration and necrosis. A tumour embolus has formed in the central vein. HE. ×100.

glands by the same neoplastic population, with associated parenchymal degeneration or necrosis. Neoplastic cells were present in the spleen and lymph nodes, and in both tissues there was atrophy of lymphoid follicles. Sternal and femoral bone marrow contained many abnormal blast cells, with a marked reduction in normal haemopoietic tissue.

The neoplastic cells were generally poorly differentiated, with a medium-sized round nucleus, dense nuclear chromatin and either scant or abundant cytoplasm. Some larger cells had oval or pleomorphic macronuclei, whilst others were multinucleated, with a lower nuclear to cytoplasmic ratio (Fig. 2). There

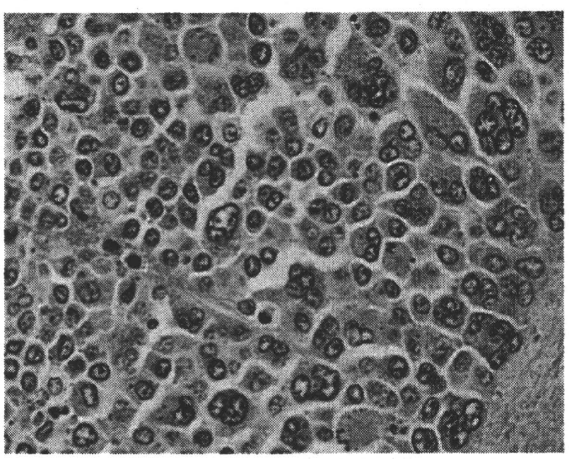

Fig. 2. Neoplastic cells within the liver. These vary greatly in size and are generally 2–3 times larger than normal lymphocytes. The majority of cells have large, round to oval nuclei with stippled chromatin and abundant cytoplasm. Some cells have macronuclei and others are multinucleated, with a lower nucleus to cytoplasmic ratio. HE. ×400.

were numerous abnormal mitoses. The majority of blast cells did not stain with PAS, but occasional individual cells were weakly stained. Masson's trichrome staining demonstrated severe fibrosis of the bone marrow (Fig. 3).

To further identify the neoplastic cells, immunohistochemical studies of femoral bone marrow, liver and kidney were performed. Sections were dewaxed, pre-treated with 0.5% H_2O_2 in methanol and then subjected to antigen retrieval with citric acid buffer (pH 6.0) and heating in an autoclave for 10 min at 121°C. Sections were then incubated with primary antibody overnight at 4°C. The primary antibodies employed were rabbit polyclonal antibodies specific for myeloperoxidase (Novocastra Laboratories, Newcastle, UK; 1 in 150 dilution); Von Willebrand Factor (Dako Cytomation, Denmark; 1 in 400 dilution); CD3 (Dako, 1 in 100 dilution); lysozyme (clone EC 3.2.1.17, Dako; 1 in 400 dilution) and monoclonal mouse antibodies specific for CD235a (clone JC159, Dako; 1 in 200 dilution); CD61-IIIa (cloneY2/51, Dako; 1 in 100 dilution); CD20 (clone L26, Dako; 1 in 100 dilution); HLA-DR alpha-chain (clone TAL.1B5, Dako; 1 in 40 dilution) and CD68 (clone KP1, Dako; 1 in 100 dilution). Following brief washes with buffer, the sections were incubated with the EnVision™ + Dual Link-HRP system (Dako) as secondary stage for 30 min. Labelling was "visualized" by treating the sections with the chromogen 3-3′-diaminobenzidine tetroxide (Dojin Kagaku, Japan) and H_2O_2. The sections were then counterstained with haematoxylin.

The majority of the neoplastic cells had granular cytoplasmic expression of the megakaryocyte-associated antigens Von Willebrand Factor (Fig. 4) and CD61-IIIa, but were negative for all other markers. On the basis of these findings, a diagnosis of AMKL (M7)-like disease with myelofibrosis was made. The neoplastic cells in AMKL often have granular cytoplasmic PAS staining when examined in a blood or a bone marrow smear (Wu *et al.*, 1996; Shukla *et al.*, 2004). The absence of significant PAS staining in the cells of the present case may relate to the formalin fixation process.

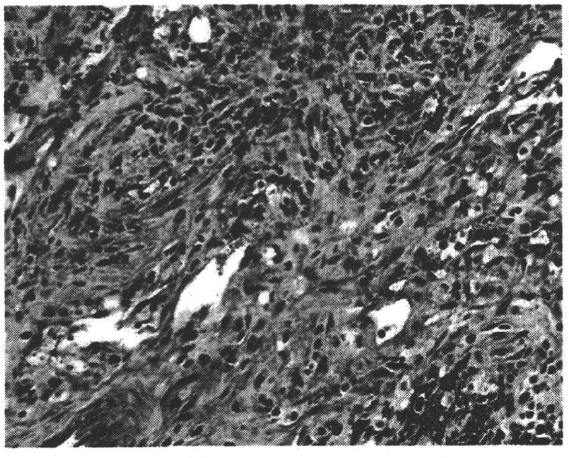

Fig. 3. Marked fibrosis (blue staining) is present within the bone marrow and admixed with neoplastic cells. Masson's trichrome. ×200.

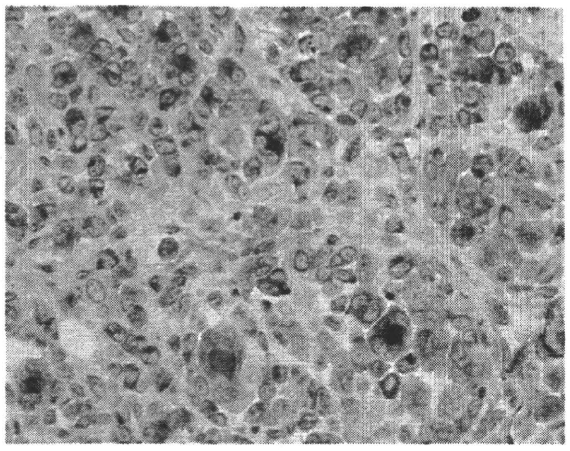

Fig. 4. Expression of Von Willebrand Factor by neoplastic cells within the bone marrow. IHC. ×400.

AMKL was first described as a subtype of acute myeloid leukaemia (AML) (von Boros and Korenyi, 1931) and was incorporated into the French–American–British (FAB) classification of AML as M7 (Bennett *et al.*, 1985). AMKL is rare, accounting for 3–5% of all human AML (Brunning *et al.*, 2001), but there is a higher incidence in children, partly due to an association with Down's syndrome (Athale *et al.*, 2001; Paredes-Aguilera *et al.*, 2003). Although AMKL is well characterized in man (Koike, 1984; Akahoshi *et al.*, 1987), in animals it has been reported only in the dog and cat (Colbatzky and Hermanns, 1993). Disrupted haematopoiesis leads to cytopenia, particularly thrombocytopenia, which becomes manifest as cutaneous petechiae, epistaxis and bleeding gums. In leukaemic patients there is often elevation of serum LDH concentration (Ferrara and Mirto, 1996). Since megakaryocytes, which store various growth factors in their alpha granules, are known to be involved in the pathogenesis of myelofibrosis, AMKL is frequently accompanied by myelofibrosis (Terui *et al.*, 1990). AMKL typically has a more guarded prognosis than other types of leukaemia (Athale *et al.*, 2001).

Differential diagnoses for AMKL include minimally differentiated AML (M0), pure erythroid

leukaemia (M6b) and acute lymphocytic leukaemia (ALL). M0, M6b and ALL are all generally negative for expression of myeloperoxidase in immunohistochemistry (IHC), as is AMKL. The neoplastic cells in AMKL occasionally have a lymphoblast-like appearance similar to M0 and ALL (Brunning *et al.*, 2001). Furthermore, neoplastic multinucleate cells are observed in both M6b and AMKL, and are often positively stained by PAS (Brunning *et al.*, 2001). Megakaryoblasts do not express myeloperoxidase, but are labelled by one or more of the megakaryocyte-associated antigens CD41, CD61 and Von Willebrand Factor (Brunning *et al.*, 2001; Daniel and Arber, 2001). The cytological and immunohistochemical features of the neoplastic population in the present case were not consistent with M0, M6b or ALL.

Further differential diagnoses for AMKL with myelofibrosis, as described in the present case, include acute panmyelosis with myelofibrosis (APMF), blastic transformation of chronic myeloid leukaemia (CML) or idiopathic myelofibrosis (IMF). APMF is characterized by multi-lineage myeloid proliferation, with a less numerous population of blast cells than in acute megakaryoblastic leukaemia (Orazi *et al.*, 2005). The cells in APMF do not express megakaryocyte-related antigens, which is inconsistent with the findings in the present case. CML is a clonal bone marrow stem cell disorder with proliferation of mature granulocytes (Travis *et al.*, 1987; Bourantas *et al.*, 1998) whereas IMF is a clonal myeloproliferative disorder that is characterized by abnormal deposition of collagen within the bone marrow (Hirose *et al.*, 2001). Human patients with CML or IMF also develop terminal blastic transformation, and these blast cells have frequently been identified as megakaryoblasts (Travis *et al.*, 1987; Bourantas *et al.*, 1998; Hirose *et al.*, 2001). Although the present case most likely represents AMKL with myelofibrosis, it is difficult to entirely exclude the alternative interpretation of blastic transformation of CML or IMF. For this reason, the present case has been described as an AMKL (M7)-like disease.

To our knowledge, this is the first case of spontaneously arising AMKL-like disease in non-human primates. The affected monkey had SRV/D infection, which may have contributed to the development of the neoplastic disease (Guzman *et al.*, 1999). Alternatively, a genetic mechanism may be proposed as humans with Down's syndrome have predisposition to the development of AMKL associated with a somatic mutation in the gene encoding the GATA1 transcription factor protein (Shimizu *et al.*, 2008). Further cases of such leukaemia in non-human primates should be subject to genetic investigation.

Acknowledgments

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Maternal Behavior of Laboratory-born, Individually Reared Long-tailed Macaques (*Macaca fascicularis*)

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To investigate maternal behavior in laboratory-born, individually reared monkeys, we carried out a statistical analysis of 896 long-tailed macaques (*Macaca fascicularis*) based on breeding records of the Tsukuba Primate Research Center (National Institute of Biomedical Innovation, Ibaraki, Japan). Data were obtained from 3266 cases of normal delivery between 1982 and 2004. In each case, maternal behavior was classified as either adequate or inadequate. We examined the effects of parity and the sex of the infant on maternal behavior. We also investigated the similarity of maternal behavior between mothers and their daughters and the effect of quality of maternal care received in infancy on maternal behavior as an adult. The results showed that only the mother's number of deliveries had a significant effect on maternal behavior. The greatest improvement of maternal behavior was observed at second delivery, and the incidence of improvement kept being above 0 thereafter. Our results suggest that, as reported previously, parity is an important factor in the adequacy of maternal behavior in individually reared monkeys.

Abbreviations: IR, improvement ratio; IA, individual adequacy; SPF, specific pathogen-free; TPRC, Tsukuba Primate Research Center

The Tsukuba Primate Research Center (TPRC; National Institute of Biomedical Innovation, Ibaraki, Japan) was established in 1978. The original purpose of monkey breeding in the TPRC was to supply the laboratory-bred monkeys for national vaccine safety testing performed in the National Institute of Infectious Diseases. All monkeys in the TPRC breeding colony were free from measles virus by 1982, and the breeding colony became free *Shigella, Salmonella, Mycobacterium tuberculosis, Mycobacterium bovis*, simian varicella virus, and herpes B virus by 2002.

The TPRC now has expanded its function to provide monkeys to medical researchers in a broad range of fields, such as infection and hyperimmunization restraint. These research areas often need monkeys that are specific-pathogen free (SPF) at its highest level. To effectively maintain and supply SPF monkeys and monkey fetuses that are appropriate for each experiment, the TPRC uses individual rearing. Monkeys are separated from their mothers 5 to 6 mo after birth and receive pair-rearing with an age-matched cagemate until 2 y of age. Thereafter, they are kept in individual cages except during mating periods. Even though TPRC monkeys always have visual, auditory, and olfactory contact with their conspecifics living in the same room, their social experience is quite limited compared with that of wild or group-reared captive monkeys.

Many previous studies indicated that monkeys with limited or no social experience show abnormal social behavior. The most extreme cases are reported in a particular series of studies.[1,7,8,19,20] For the duration of their infancy, monkeys were housed without their mothers and with little or no opportunity to interact with other monkeys. As a result, they had difficulty forming normal social relationships in many interactive situations, such as playing,[1,8,20] mating,[7] and mothering.[1,7,20] The abnormal maternal behavior of these monkeys evoked interest among researchers. Some of the females that had poor social experiences as infants abused or neglected their own infants, in some cases so severely that the infants died.[20]

Many of the cited studies were observations of monkeys reared under more severe circumstances than those in a normal breeding colony. Even in normal breeding colonies, most researchers observe only a portion of the colony animals.[12,14-17,21] No large-scale investigation has examined the effects of individual rearing on the maternal behavior of the monkeys. Here we report the statistical analysis of data accumulated over a period of 20 y on the maternal behavior of more than 3000 cases involving approximately 900 monkeys that were individually reared for most of their lives.

Materials and Methods

Rearing and breeding conditions of the TPRC. The rearing and breeding conditions of the TPRC have changed somewhat over time, depending on prevailing regulations and practices. However, what has never changed is the individual rearing of monkeys and the length of time during which monkeys are reared with their mothers or age-mates.

The rearing and breeding conditions were fixed as follows from 2005. Monkeys in the TPRC breeding colony are reared in individual cages (0.5 m wide × 0.8 m high × 0.9 m deep; stainless steel mesh). The breeding rooms are rectangular, and the individual cages are installed on the long sides of the room. Each room contains at least 90 cages. Most (90% to 95%) cages are occupied continuously. Therefore, monkeys can always make visual, auditory and olfactory contact with their room-mates. Ambient temperature in the rooms is kept about 25 °C, and humidity is set at 50% to 60%. The air is replaced 12 times hourly.

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Lighting is on for 12 h, from 0700 to 1900. Monkeys are provided with apples in the morning, and monkey chow is given to them twice in the afternoon. Water is available ad libitum.

Monkeys in the breeding colony are inspected daily by experienced animal technicians. When any abnormality is found, a veterinarian examines the monkey promptly and applies the appropriate treatment. Moreover, the monkeys are medically examined under anesthesia at least once every 2 y. The medical examination consists of body weight measurement, tuberculin test, blood sample, stool test, examination of the fundus, and a medicated bath.

Monkeys are separated from their mothers 5 to 6 mo after birth and receive peer-rearing with an age-mate until 2 y of age. After that, they are reared individually except during the mating period. The mating period begins 11 d after menstrual bleeding is observed, which is 1 d before the estimated ovulation. The monkey is anesthetized and moved to the cage next to the mating partner at least 1 d before the start of the mating period. The mating period is begun by removing a partition between the cages. After 3 d the partition is replaced.

Pregnancy diagnosis is conducted by the ultrasonography under anesthesia 5 wk after the end of the mating period. If the female is not pregnant, she begins the next mating period around the presumed ovulation day. During pregnancy, females are reared individually. After delivery, the dam and her infant are reared together for about 6 mo, after which they are separated. The offspring begin peer-rearing with an age-mate, and dams are reared individually again.

These rearing and breeding conditions are approved by the Institutional Animal Care and Use Committee of National Institute of Biomedical Innovation, Japan.

Laboratory procedures. Subjects were 896 laboratory-born long-tailed macaques (*Macaca fascicularis*) in the breeding colony of the TPRC. Data were obtained from breeding records collected between March 1982 and March 2004. Individual monkeys gave birth from 1 to 12 times (mean ± SD, 3.7 ± 2.3). The total number of normal births was 3266.

Animal technicians determined the adequacy of maternal behavior on the basis of daily inspection from the infant's birth to separation of mother and infant, about 6 mo.

Breeding records reported whether a dam showed inadequate maternal behavior, regardless of duration or frequency. Moreover, even if a mother that was once judged to be 'inadequate' never again showed inadequate behavior to the same offspring, the record was never changed. In short, only the cases in which inadequate maternal behavior was *never* observed by the animal technicians were recorded as adequate.

Inadequate maternal behavior was defined as rejection of the infant, holding an infant incorrectly, refusal to nurse, or violence against the infant. The dam's avoidance of, or escape from, physical contact with her infant was considered rejection of the infant. Holding an infant incorrectly means that the dam held her infant on her ventral side upside down. Refusal to nurse means that the dam held her infant correctly but prevented the infant's access to her nipples. Violence against the infant was physically hitting or stepping on her offspring.

When a dam showed inadequate maternal behavior, she and her infant were separated, and food and medical treatment were given to the infant as needed. If the infant regained his or her health, he or she was returned to the mother. In most cases, the infant was returned to the dam only once. The animal technician observed the pair for about 10 min, and if the dam took care of her infant normally, the infant was allowed to remain. If inadequate maternal behavior was observed at that time or at the inspection thereafter, the infant was separated from the mother again and was never returned to her.

If the infant was in poor physical condition for more than 2 to 3 d, or if the dam showed inadequate mothering after the infant's return, the infant was reared by artificial nursing or by foster mothers.

Consistency in adequacy of maternal behavior. Multiparous monkeys were classified into 4 groups based on changes in their adequacy of maternal behavior: good, improved, poor, and inconsistent. Monkeys that showed adequate care for all their offspring were classified as having good maternal behavior. The monkeys belonging to the improved group showed inadequate maternal behavior at the first delivery or between the first and a certain delivery number, but they never had inadequate behavior thereafter. Note that our definition of improvement is based on the change in maternal behavior between deliveries, not on a change with the same infant. Monkeys that showed inadequate maternal behavior with all their infants were classified as having poor maternal behavior. The remaining monkeys were classified as having inconsistent maternal behavior.

Primiparous monkeys were classified into 2 groups (good and poor), based on their maternal behavior.

Effects of parity on maternal behavior. To determine whether adequacy of maternal behavior improves with increasing parity, we compared the proportion of 'adequate care' cases at each number of deliveries. As shown in Figure 1, adequacy of maternal behavior appears to improve after giving birth at least twice. However, this analysis may not reflect the natural tendency for improvement of maternal behavior, as monkeys showing inadequate maternal behavior may have been preferentially been excluded from the breeding colony as a means of colony management. Therefore, in a second analysis, we evaluated dams that gave inadequate maternal care to their first offspring based on the adequacy of mothering toward the second offspring. The monkeys that did not improve their maternal behavior at the second delivery were classified again, this time according to their maternal adequacy toward the third offspring. Data from the inconsistent group were excluded from this analysis. We then calculated the improvement ratio (IR) for each delivery number as $N_a / (N_a + N_i)$, where N_a is the number of monkeys that improved their maternal behavior and N_i is the number of monkeys that continued inadequate maternal behavior. If all monkeys that showed inadequate mothering toward the previous offspring improved, the value of IR was 1. Conversely, if none improved, the value of IR was 0.

Relationship of the maternal behavior of the mother to that of the daughter. A total of 340 pairs of mothers and daughters among the monkeys were included in the study. Because the subjects in the present study consisted of the monkeys belonging to various generations, some subjects were represented both as mother and as daughter. When a monkey had several daughters who experienced delivery, the dam was paired with each daughter. In other words, the number of times that a monkey was represented as mother was the same as the number of her daughters who had experience of delivery. Two analyses were conducted using the data from these pairs..

First, to investigate similarity of maternal behavior between mother and daughter, we calculated the ratio of the number of observations of adequate maternal behavior to the total number of deliveries for each monkey. We defined this value as individual adequacy (IA). We then examined the correlation of IA between daughter and mother by Spearman rank correlation coefficient.

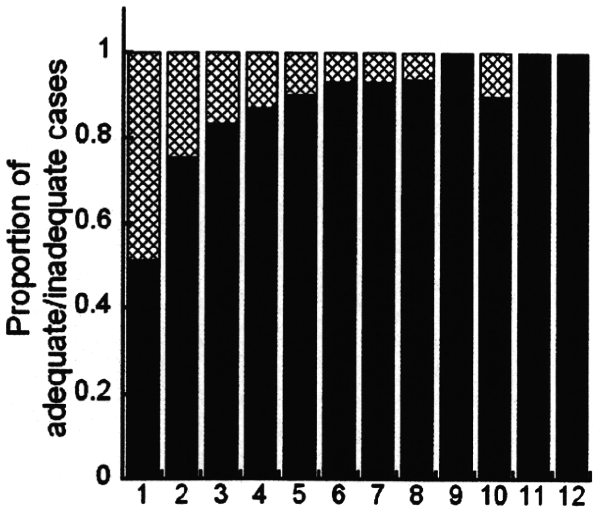

Figure 1. Apparent improvement of maternal behavior with childbirth. We classified each case by the number of deliveries and calculated the ratio of adequate to inadequate maternal behavior. Because there was a possibility that monkeys that showed inadequate behavior were excluded preferentially from the breeding colony, we conducted another analysis (see Figures 2 and 3).

Second, to investigate whether the quality of maternal care received in infancy influenced the maternal behavior of the daughter, we divided the daughters into 2 groups according to the quality of maternal care they received (adequate and inadequate). We then compared the IA value and the consistency in adequacy of maternal behavior in the 2 groups of daughters by *t* test.

Effects of sex of the infant on maternal behavior. We divided all cases by the sex of the infant. We then calculated the incidence of adequate and inadequate maternal behavior in each group and compared them by χ^2 test.

All statistical analyses were performed using StatView statistical software (version 5.0; SAS Institute, Inc., Cary, NC).

Results

Consistency in adequacy of maternal behavior. Of 3266 normal deliveries, 2459 (75.3%) were associated with adequate maternal behavior, and the remaining 807 (24.7%) were categorized as having inadequate maternal care. The severity and continuity of inadequate maternal behavior differed. As soon as inadequate behavior was observed by an animal technician, the infant was separated from the mother for treatment as needed. In 390 of the 807 (48.3%) cases with inadequate maternal behavior, the physical condition of the infant was good, and the dam showed signs of accepting her infant; therefore, the infant was returned to his or her biological mother and was reared by her until their separation. However, if the mother continued displaying inadequate behavior or if the physical condition of the infant was impaired, the infant was reared by artificial nursing (266 of the 807 cases, 33.0%) or by foster mothers (151 cases, 18.7%).

The results of classifying monkeys by the consistency in adequacy of maternal behavior are shown in Table 1. Of primiparous monkeys (n = 187), 42.2% were categorized as having good maternal behavior, and 57.8% were categorized as having poor maternal behavior. Of multiparous monkeys (n = 709), 52.0% were categorized as good; most of the remaining monkeys had

Table 1. Distribution of monkeys, arranged by the consistency of adequacy of maternal behavior

Parity	No. of subjects				
	Good	Improved	Poor	Inconsistent	Total
1	79	na	108	na	187
2	77	30	44	2	153
3	56	58	29	6	149
4	67	34	17	5	123
5	56	24	11	3	94
6	43	22	0	7	72
7	32	15	2	5	54
8	20	11	3	1	35
9	12	7	0	0	19
10	5	2	0	1	8
11	0	1	0	0	1
12	1	0	0	0	1
Primiparous	79	na	108	na	187
Multiparous	369	204	106	30	709
Total	448	204	214	30	896

na, not applicable

maternal behavior that improved with subsequent births (28.8%) or that remained poor regardless of parity (15.0%). Only 4.2% of the multiparous monkeys were classified as having inconsistent maternal behavior regardless of parity.

In the improved group, the number (mean ± SD) of times that dams continued to show inadequate behavior from the first delivery was 1.5 ± 0.9, with 4.5 ± 2.1 deliveries. The total number of normal deliveries which dams in this group experienced was 909. In 614 of these cases, their maternal behaviors were determined as adequate. In the other 295 cases, the maternal behaviors were determined as inadequate. In the inconsistent group, the mean number of deliveries was 5.1 ± 1.9, and the total number of normal deliveries was 152. In 84 of these cases, maternal behavior of the dams was determined as adequate. In the other 68 cases, the maternal behavior was determined as inadequate.

Effects of parity on maternal behavior. As shown in Figures 2 and 3, the incidence of adequate maternal behavior at the first delivery was 51.7%. The highest IR was observed at the second delivery. After that, the value of IR was low but nonvanishing (Figure 3). However, only a few dams required more than deliveries to develop adequate maternal behavior (Figure 2).

Relationship of the maternal behavior of a mother to that of her daughter. The correlation between the IA of mothers and daughters was not statistically significant (Figure 4; Spearman's rank correlation coefficient, n = 340 pairs, $P > 0.1$). Furthermore, the IA value of the daughters that received adequate maternal care in infancy was not significantly different from that of the daughters that received inadequate care in infancy (*t* test, $t(338) = -0.1$, $P > 0.10$; IA of daughters that received adequate care, 0.5 ± 0.4; IA of daughters that received inadequate care, 0.5 ± 0.4). Moreover, consistency in adequacy of maternal behavior did not differ between the 2 groups of daughters (Figure 5).

Effect of sex of the infant on maternal behavior. In total, 1709 infants were male and 1557 were female. For 75.0% (1282) of the male infants and 75.6% (1177) of the female, mothers showed adequate maternal behavior. The quality of maternal care the infants received did not differ according to their sex (χ^2 test, $P > 0.1$).

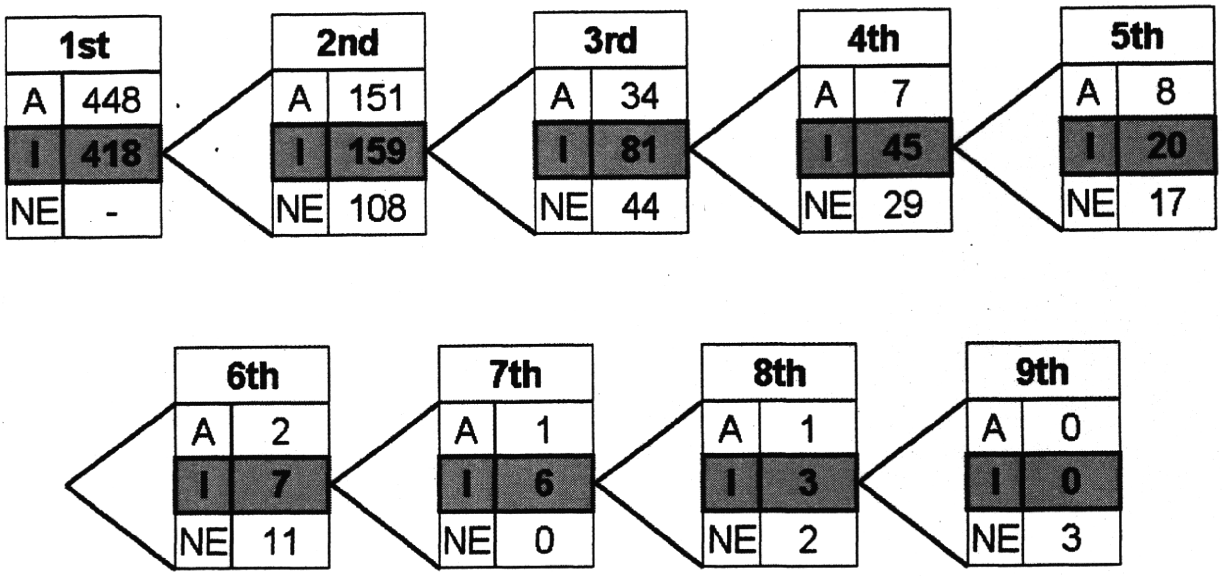

Figure 2. Schema for calculation of improvement ratio (IR) for each delivery number. The ordinal number at the top of each box is the number of deliveries. Inside each box is the number of subjects in each category: A, monkeys that showed *adequate* maternal behavior at delivery; I, monkeys that showed *inadequate* maternal behavior at delivery; NE, monkeys that *did not experience* that number of deliveries. The total number of monkeys at each delivery number is equal to the number of monkeys categorized as I at the previous delivery number. Because the number of monkeys belonging to I became 0 at the ninth delivery, we stopped the analysis at that step.

Discussion

One of the most important questions we asked is whether monkeys in our breeding colony show inadequate maternal behavior at a higher rate than do group-reared or free-ranging monkeys. The incidence of inadequate maternal behavior in the TPRC was 24.7% (807 of 3266 cases). However, our assessment of inadequacy is quite strict: a dam defined as having inadequate maternal performance as soon as she displays a single suboptimal rearing behavior. Moreover, this classification is never revised, even if the dam improves her maternal behavior by the time of protocol-defined separation from her infant. In approximately half (390 of 807) of the cases defined as having inadequate maternal behavior, the dam showed the suboptimal behavior only transiently and then reared her infant successfully without any intervention until the separation. Therefore, we consider the actual percentage of inadequate maternal behavior to be 12.8% (417 of 3266 cases).

In 1 study, the incidence of inadequate maternal behavior in group-reared monkeys was 1.9% to 12.2%.[13] However, the authors stated that the actual incidence might be higher because they counted only severe cases of neglect and abuse. We are aware that not all infant deaths are the result of inadequate maternal care, and for comparison, infant mortality in a free-ranging troop was 6.7% to 16.2%.[9,10,18] Compared with these data, the incidence of inadequate maternal behavior in the TPRC is not unreasonably high.

However, our results do not suggest that the social experience of dams has no effect on their maternal behavior. In our study, the incidence of adequate maternal behavior by individually reared monkeys at the first delivery was 51.7%. In a 1981 study,[21] among 6 long-tailed macaques that received peer-rearing with 6 to 7 age-mates until sexual maturation, all but 1 showed normal maternal care to the first offspring. In a 1989 study of 10 long-tailed macaques reared in different social conditions,[11] 1 group consisted of monkeys that were born and reared in family groups until the first delivery. Another group consisted of monkeys that were born in family groups but were reared with 6 to 7 age-mates. All monkeys in both groups showed adequate maternal behavior. In other studies,[1,20] most of the monkeys that had limited social experience could not rear their first infants, but these authors observed only 4 to 8 monkeys. Of 50 rhesus monkeys that were reared without mothers, 34 (68.0%) abused or neglected their first infants.[19]

The monkeys that showed increased incidence of adequate maternal behavior[11,21] than that in the current study received peer-rearing for a longer time and with more age-mates than did those in the TPRC. In contrast, those that showed poorer rearing skills[1,19,20] were reared in a poorer social environment than those in the TPRC. These results suggest that the duration and complexity of social rearing affect maternal behavior at the first delivery.

As already mentioned, about half the monkeys in our study showed inadequate mothering at their first delivery, but about half of those monkeys improved their maternal behavior by their second delivery. Moreover, few monkeys showed inconsistent maternal behavior overall (Table 1). Overall, about 75% of the monkeys we studied had developed adequate maternal behavior by the time of their second delivery. These results suggest that the maternal behavior can be improved through the delivery experience. Regardless of whether the monkeys are captive or wild, many investigators have suggested that previous deliveries help the dam learn how to treat her infant and contribute to the overall improvement of infant survival rates.[6,9,10,13-15] Even monkeys that received no maternal care in infancy were able to improve their maternal behavior with repetition of delivery.[7,19]

We identified no factors other than parity that affected maternal behavior. Previous studies in group-living monkeys suggested that infant abuse, not neglect, tended to be observed only in particular matrilines.[12,13] We cannot know all the details regarding inadequate maternal behavior at the TPRC in the past, because the breeding records were designed to record

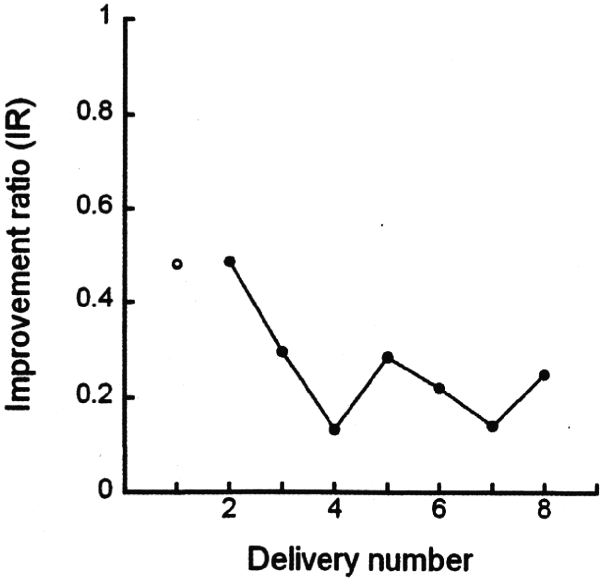

Figure 3. Improvement ratio (IR) for each delivery number. The open circle indicates the incidence of adequate maternal behavior at the first delivery for all subjects (n = 896). The closed circles indicate the IR. We calculated the IR for each delivery number by the formula N_a / (N_a + N_i), where N_a is the number of monkeys that improved their maternal behavior, and N_i is the number of monkeys that continued inadequate maternal behavior. The actual values of N_a and N_i for each delivery number are shown in Figure 2.

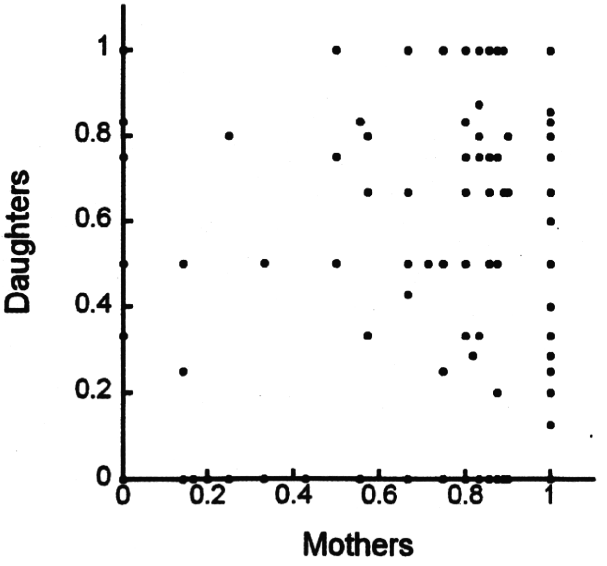

Figure 4. Correlation of individual adequacy (IA) between mothers and daughters (n = 340 pairs). Because some dots overlapped, the number of dots in the figure is less than the number of the pairs. There was no significant relationship between the IA of mothers and that of daughters.

maternal behavior only as adequate or inadequate. Perhaps we found no correlation in the adequacy of maternal behavior between mothers and daughters because of a limited choice of categories. We would need to examine the medical history for each infant until mother–infant separation to determine why maternal behavior was judged inadequate (for example, if the dam physically abused her infant). Moreover, although infant

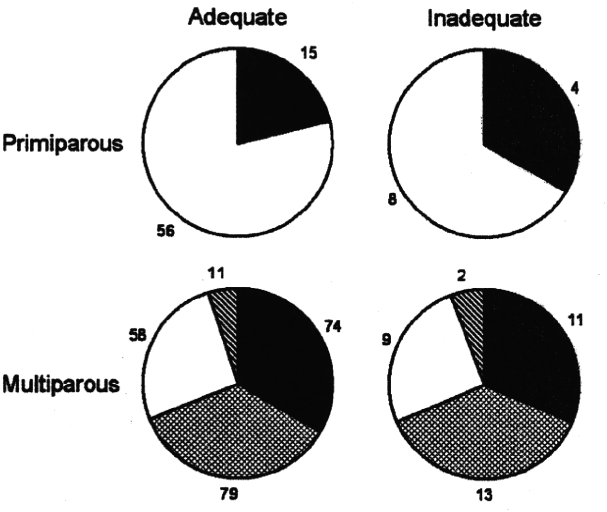

Figure 5. Relationship of maternal behavior of the mothers to that of their daughters. We divided the daughters into 4 groups according to the quality of maternal care they received (adequate or inadequate) and parity (primiparous or multiparous). Numbers show the number of subjects in each group. Black, good; cross-hatched, improved; white, poor; striped, inconsistent.

behavior between the sexes is quite different,[3,16] we did not find any effects of the infant's sex on maternal behavior. Supporting our finding, another study reported that sex of the infant was not a risk factor for abuse or neglect.[15]

Few previous reports about maternal behavior in long-tailed macaques are available; to our knowledge, there are only 2 observational experiments that compared maternal behavior in different rearing conditions.[11,17] Although another article[21] contains statistical data on maternal behavior in captive and peer-reared long-tailed macaques, only 6 subjects were assessed at their first delivery only. Most of the other studies we cite involve other *Macaca* species, and interspecies differences might not be negligible. Considering that long-tailed macaques are used in experiments as widely as other species, more information about their maternal behavior is needed.

Although the incidence of inadequate maternal behavior in the TPRC is not unreasonably high, we are apprehensive about the future. The maternal behavior of peer-reared or isolation-reared monkeys may differ from that of mother-reared monkeys,[4] and differences in maternal behavior may exist between laboratory-born and wild-born monkeys in the TPRC.[17] The differences might increase in each subsequent generation, as could the incidence of inadequate maternal behavior. Indeed, in the current study, the proportion of daughters in the group with good maternal behavior was lower than that overall and the ratio of the daughters in the group with poor maternal behavior was higher than that overall (Table 1 and Figure 5). Directly comparing the adequacy of maternal behavior between generations is difficult because the mean number of deliveries of each generation appears to differ. However, we need to determine whether the incidence of inadequate maternal behavior is likely to increase in future generations. Moreover, the SPF level required differs by experiment, and whether the level of social experience for each subgroup of monkeys can be changed depending on the purpose of the proposed research will be important to determine. Perhaps, for example, extension of the pair-rearing period[2] will decrease incidence of abnormal behavior in our monkeys. Further, modification of the cages may

make it possible to avoid disturbing the physical contact among the monkeys living in the adjoining cages.[5] For TPRC—and other facilities—to continue supplying high-quality monkeys, such possibilities need to be considered.

Acknowledgments

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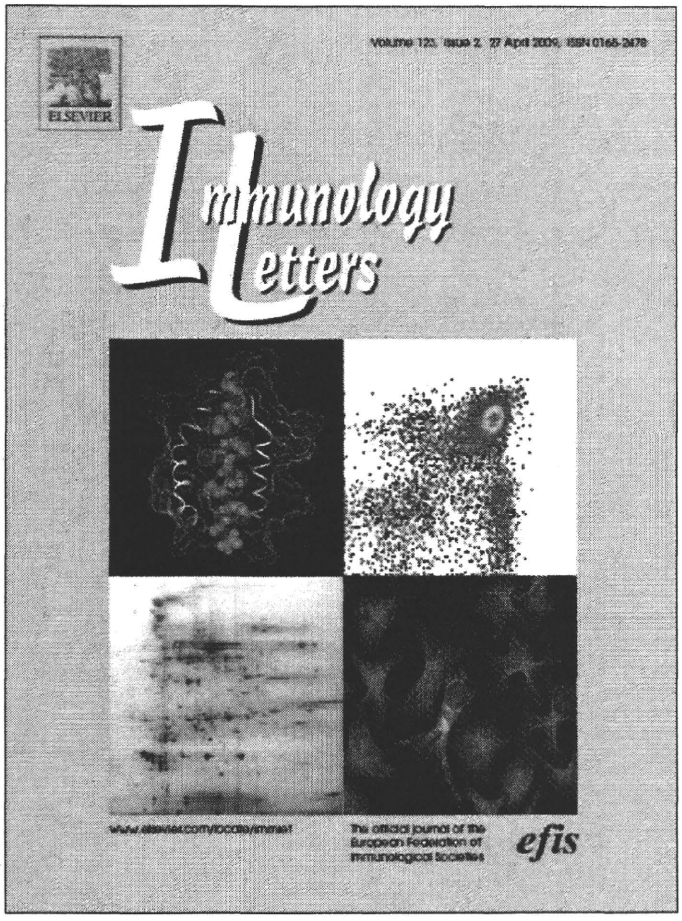

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Expression of IL-5Rα on B-1 cell progenitors in mouse fetal liver and involvement of Bruton's tyrosine kinase in their development

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ABSTRACT

B-1 cells are a subset of B cells responsible for the production of natural antibodies. Although the amount of natural antibody is tightly regulated, how this regulation occurs remains unknown. We examined the expression of IL-5 receptor, a cytokine receptor critical for homeostatic proliferation of B-1 cells, on B-1 cell progenitors in the fetal liver. We identified B-1 progenitors expressing low levels of IL-5 receptor α chain (IL-5Rα) and eosinophil progenitors expressing higher levels of IL-5Rα in the fetal liver. Moreover, the number of these B-1 progenitors were significantly reduced in the fetuses of mice deficient in Bruton's tyrosine kinase (Btk), even though IL-5 and thymic stroma lymphopoietin signaling are intact in early B lineage cells in Btk-deficient mice. These data suggest that IL-5 is possibly involved in B-1 cell development and an uncharacterized, Btk-dependent regulatory signaling pathway is involved in unexpectedly early stages of B-1 cell differentiation.

1. Introduction

Vertebrates are protected from pathogens by two arms of the immune system: innate and acquired immunity. The innate immune system relies primarily on various pattern-recognition receptors such as the family of Toll-like receptors. Since this type of receptors is readily expressed and can sense a wide variety of pathogens, the innate immune system is activated immediately upon pathogen entry. In contrast, activation of the acquired immune system requires time to select and expand antigen-specific T and B cells. With its high specificity for antigen and long-lasting memory, activation of the acquired immune system is important for complete elimination of pathogen and immunity to subsequent infections by the same pathogen. A certain level of immunoglobulins exist in the body independent of infection, and these are designated natural antibodies. These natural antibodies are produced by a special subset of B lymphocytes called B-1 cells [1]. B-1 cells are distinguished from conventional B cells (B-2 cells) by their high expression of IgM, low expression of IgD, expression of CD43, and lack of CD23 and CD21 expression. These cells can be further sub-divided into $CD5^+$ B-1a cells and $CD5^-$ B-1b cells. By their preformed reactivity to various pathogens, natural antibodies can be included in the innate immune system. In fact, X-linked immunodeficiency (XID) mice deficient in B-1 cells and natural antibodies are more susceptible to *Streptococcus pneumoniae* infection than wild-type mice, and the wild-type susceptibility can be restored to XID mice by administration of normal mouse serum [2]. In addition, recent studies have shown that B-1 cells and natural antibodies are indispensable for effective induction of certain acquired humoral immune responses [3] and cellular immune responses [4]. Despite their importance in innate and acquired immunity, the differentiation pathway of B-1 cells is poorly understood. One hypothesis is that B-1 cells are an activated form of B-2 cells, and that this activation occurs through B cell receptors that have certain specificity like to phosphatidylcholine. This hypothesis is supported by the observation that in the trasngenic mice of B cell receptor cloned from B-1 cell, all the B cells show a B-1 phenotype [5]. Another hypothesis is that B-1 cells arise from progenitors other than progenitors for B-2 cells. This idea stems from studies showing that transfer of adult bone marrow into irradiated recipient mice resulted in repopulation of B-2 but not B-1 cells, whereas fetal liver cells gave rise to both B-1 and B-2 cells in the same situation [6,7]. Consistent with the latter hypothesis, B-1 lineage-committed progenitors were recently identified as $CD19^+$ $B220^-$ cells in fetal and juvenile mouse bone marrow [8]. These "B-1 progenitors" display a phenotype of proB cells and are responsive to thymic stroma lymphopoietin (TSLP).

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However, regulatory mechanisms controlling their differentiation or origin of these B-1 cell progenitors are still unknown.

Interleukin-5 (IL-5) was originally identified as a soluble factor secreted from activated T cells that can promote antibody secretion from B cells [9]. Its receptor consists of a ligand-specific α chain (IL-5Rα/CD125) and signal-transducing β chain (βc/CD131) that is shared among IL-3 receptor and GM-CSF receptor [10]. While expression of IL-5Rα on B-2 cells is limited to activated cells, most B-1a and B-1b cells constitutively express IL-5Rα [11]. Furthermore, IL-5 knock-out mice, IL-5Rα knock-out mice, and mice injected with anti-IL-5 neutralizing mAb show a reduction in number and size of B-1 cells [12–14]. These data suggest that IL-5 signaling is closely related to the differentiation and maintenance of B-1 cells. Although many IL-5-dependent proB or preB cell lines have been established, no *in vivo* counterpart of such cells have been described, nor has a role(s) for IL-5 in early B cell development been elucidated [15,16]. In this study, we examined the B-1 cell progenitor activity of fetal liver cells expressing IL-5Rα and the signaling requirements for B-1 cell progenitors.

2. Materials and methods

2.1. Mice

C57BL/6J, BALB/cA, BALB/cByJ, and C.B-17/Icr-scid/scid (SCID) mice were purchased from the Japan CLEA company (Shizuoka, Japan). IL-5Rα knock-out mice were previously described [13]. Btk knock-out mice were provided by Dr. F.W. Alt (Howard Hughes Medical Institute, The Children's Hospital, Boston, MA) [17]. C57BL/6.*xid* mice were provided by Dr. A. Singer (National Institute of Health, Bethesda, MD). All mice were housed and maintained in the Laboratory Animal Research Center, the Institute of Medical Science, the University of Tokyo or Research Animal Facility of National Institute of Biomedical Innovation. Fetuses were obtained from pregnant mice 15 days after vaginal plugs were observed. Due to the genetic background of IL-5Rα knock-out mice (C57BL/6), Btk knock-out mice (C57BL/6) Xid mice (C57BL/6) and SCID mice (BALB/c congenic), either C57BL/6 or BALB/c mice were selected in each experiment. $CD19^+$ $B220^-$ cells were similarly observed in both strains, although BALB/c strain tends to have higher number of these cells (data not shown). All experiments were operated under the guidelines of animal use at University of Tokyo and National Institute of Biomedical Innovation.

2.2. Antibodies and reagents

Monoclonal antibody (mAb) against IL-5Rα (clone H7) was purified from ascitic fluid of BALB/c nu/nu mice that had been injected with a corresponding hybridoma. Purified mAb was biotinylated using NHS-biotin (Pierce/Thermo Fisher Scientific, Rockford, IL) or labeled with Alexa Fluor 647 using a monoclonal antibody labeling kit (Molecular Probes/Invitrogen, Carlsbad, CA). Anti-mouse IgM mAb (clone M41) was purified and conjugated to Fluorescein Isothiocyanate (FITC) in our laboratory. The following mAbs were purchased from BD Biosciences (San Jose, CA): FITC-conjugated CD3, CD5, Gr-1, CD43, CD45R/B220; Phycoerythrin (PE)-conjugated CD19 (1D3), CD23, CD43, CD127/IL-7Rα; biotinylated anti-IgM^a; Peridinin chlorophyll protein (PerCP)-conjugated CD45R/B220; and allophycocyanin (APC)-conjugated CD45R/B220, CD117/c-kit, and CD11b/Mac-1. FITC-conjugated TER-119, PE-conjugated CD19 (6D5) and PE-Cy7-conjugated CD45R/B220 were purchased from eBioscience (San Diego, CA). FITC-conjugated CD11b/Mac-1 was purchased from CALTAG/Invitrogen (Carlsbad, CA). Anti-JAK2 antiserum, anti-SHC antiserum, and anti-phosphotyrosine mAb were purchased from Upstate Biotechnology/Millipore (Billerica, MA). Anti-stat5 antiserum was a gift from Dr. Hiroshi Wakao (DNAX Research Institute, Palo Alto, CA). Mouse IL-5 was purified from culture supernatant of CHO cells stably transfected with an IL-5 expression vector using an affinity column to which anti-IL-5 mAb (NC17) had been immobilized [18]. Stem cell factor and IL-7 were purchased from Peprotech (Rocky Hill, NJ). Flt3-L and TSLP were purchased from R&D Systems (Minneapolis, MN).

2.3. Cell preparation, staining, and sorting

A single-cell suspension was prepared by mincing fetal livers on nylon mesh filters or flushing femurs and tibias of adult mice using a 27G needle attached to a syringe. Fetal liver cells were then depleted of erythroid cells using anti-TER119 microbeads and magnetic separation columns (LD column) with a Quadro MACS magnet according to the manufacturer's instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). In some experiments, fetal liver cell suspensions were just depleted of erythrocytes by ACK lysing buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM EDTA, pH 7.3). In the case of adult bone marrow, cells were first incubated with purified monoclonal antibodies against Gr-1, CD3, and TER-119, and labeled cells were depleted with anti-Rat IgG microbeads using LD columns with a Quadro MACS magnet. For detection of IL-5Rα$^+$ cells in the fetal liver, erythroid-depleted cells were stained with FITC anti-IgM, biotinylated anti-IL-5Rα, PerCP CD45R/B220, and APC CD19 antibodies. For the detection of IL-5Rα$^+$ cells in the bone marrow, lineage-depleted cells were stained with a cocktail of FITC-conjugated lineage marker antibodies, biotinylated anti-IL-5Rα and allophycocyanin-conjugated anti-c-kit. In either case, biotinylated anti-IL-5Rα andtibody was detected with PE-conjugated streptavidin. For detection of prolymphocytes (ProL), cells were stained with a FITC-conjugated lineage marker antibody cocktail, as well as with PE-anti-IL-7Rα and APC-anti-c-kit. For the enumeration of B-1 progenitors, fetal liver cells were stained with FITC-labeled lineage marker antibody cocktail (Gr-1, TER-119, CD3, DX5, anti-IgM), PE-anti-CD19 and PE-Cy7-CD45R/B220. Stained cells were analyzed by FACSCalibur or LSR II flow cytometer (BD Bioscience), or sorted with FACSAria cell sorter (BD Bioscience).

2.4. Cell cultures

OP9 stromal cells were maintained in α-MEM supplemented with 15% FCS and antibiotics [19]. A single-cell layer of OP9 cells was prepared in 24-well culture plates and irradiated with 30 Gy. Sorted cells were cultured for seven days on irradiated OP9 cell layers in OPTI-MEM I medium supplemented with 2% FCS, 50 μM 2-mercaptethanol (2-ME), antibiotics, and the indicated cytokines. At the end of the culture period, non-adherent cells were harvested, counted, and stained with fluorescence-labeled mAbs as noted.

2.5. Establishment of IL-5-dependent early B lineage clones

Long-term bone marrow cultures were initiated from C57BL/6J mice or C57BL/6.*xid* mice according to the protocol established by Whitlock and Witte [20]. Briefly, bone marrow cells were isolated from each femur and cultured in 7 mL of RPMI1640 medium supplemented with 5% FCS, 50 μM 2-ME, and antibiotics in a 25-cm^2 culture flask (day 0). On day 3, 3 mL of fresh medium were added to each flask. On day 7, 7 mL of medium were removed from each flask and 3 mL of fresh medium were added. On day 10, 4 mL of fresh medium were added. The same cycle of medium change was then repeated. Five weeks later, growing non-adherent cells were collected and cloned on an ST2 cell layer in 96-well, flat-bottom culture plates in the presence of 50 U/mL of IL-5 [21]. In order to generate stroma-independent clones, the resulting clones were re-cloned in the presence of IL-5 without ST2. Cloned cell lines were

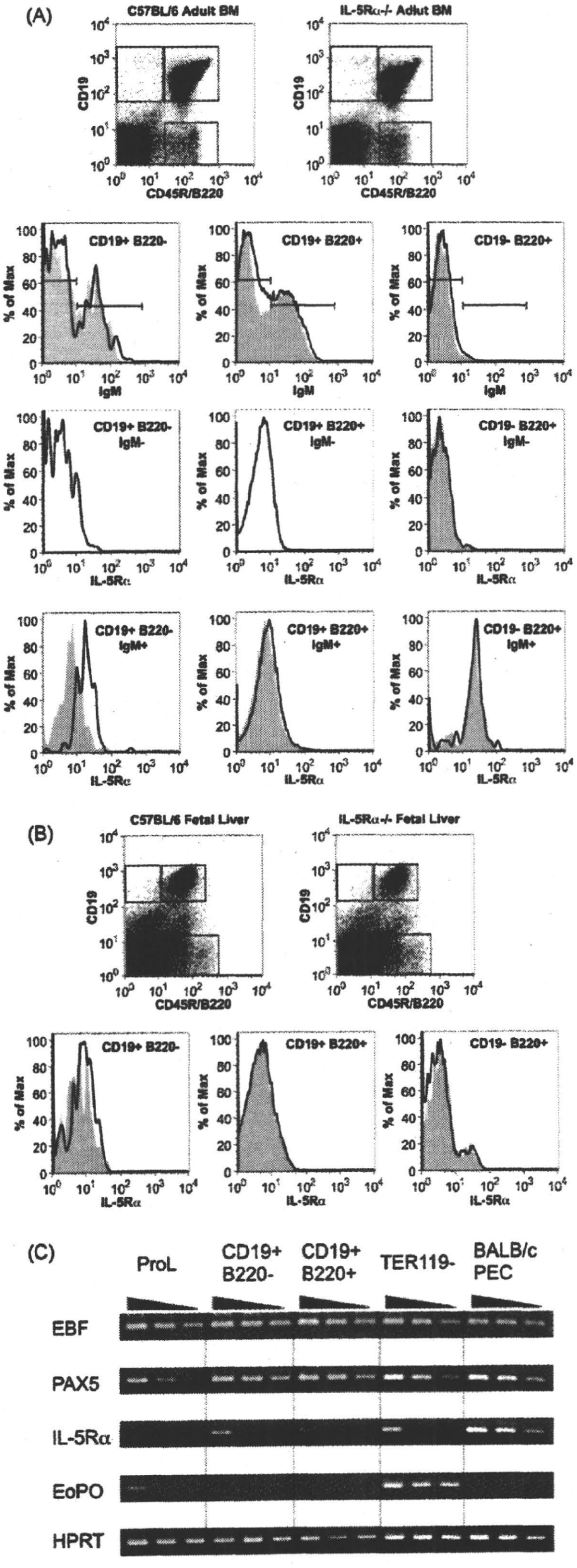

Fig. 1. CD19$^+$ B220$^-$ fetal liver cells show weak expression of IL-5Rα, which is detectable only by RT-PCR. (A) Bone marrow cells from C57BL/6J mice and negative control IL-5Rα knock-out mice were stained with antibodies against CD19, CD45R/B220, IgM, and IL-5Rα, then analyzed on a FACSCalibur flow cytometer. B lineage cells were sub-fractionated based on CD19 and B220 expression (top row), and each subset was further divided based on expression of surface IgM (second row). Staining of IL-5Rα is shown in the bottom two rows. Solid lines and filled histograms represent C57BL/6J and IL-5Rα knock-out mice, respectively. (B) Cells from day 15 C57BL/6J and IL-5Rα knock-out fetal liver were analyzed as in (A) but omitting anti-IgM staining and sub-fractionation. (C) Total RNA was extracted from sorted fetal liver cells and the expression of indicated genes was analyzed by semi-quantitative RT-PCR. ProL were purified from TER119$^-$ fetal liver cells as Lin$^-$ c-kit^{Lo} IL-7Rα$^+$ cells. CD19$^+$ B220$^-$ cells and CD19$^+$ B220$^+$ cells were sorted from TER119$^-$ fetal liver cells. TER119$^-$ cells were prepared by depleting TER119$^+$ cells from fetal liver cells using MACS beads. Total peritoneal cells from BALB/cA mice were used as a control (BALB/c PEC).

maintained in RPMI1640 supplemented with 5% FCS, 50 μM 2-ME, and 50 U/mL of IL-5.

2.6. Proliferation assay

Bone marrow-derived, stroma-independent cell lines were plated in a 96-well flat-bottom culture plate (10^4 cells per well) with 200 μL of culture medium containing various concentrations of IL-5 and then cultured for 48 h. The cells were pulse-labeled with 0.2 μCi of [^{3}H]thymidine during the last 12 h of the culture period and incorporated [^{3}H] thymidine was measured using a MATRIX 96 Direct Beta Counter (Packard Instruments, Meridien, CT).

2.7. Flow cytometric analysis of STAT5 phosphorylation

TER119$^-$ fetal liver cells or whole bone marrow cells were stimulated with 10 ng/mL IL-7 or 100 ng/mL thymic stroma lymphopoietin (TSLP) at 37 °C for 5 min in RPMI1640 medium supplemented with 8% fetal calf serum (FCS) and 50 μM 2-ME. At the end of the incubation period, cells were fixed by adding five volumes of 1% formaldehyde in 1.25x PBS at 37 °C for 10 min. Fixed cells were washed and permeabilized on ice for 30 min with Perm III solution (BD Bioscience). Then cells were stained with FITC-conjugated CD43, PE-conjugated CD19 (6D5), PerCP-conjugated CD45R/B220, and Alexa Fluor 647-conjugated anti-phospho-STAT5 (Y694) mAbs. Cells were analyzed on a FACSCalibur flow cytometer.

2.8. PCR detection of immunoglobulin gene rearrangement

Rarranged immunoglobulin genes were detected by PCR [22]. Sorted cells were lysed in PCR lysis buffer (10 mM Tris–HCl pH 8.4, 50 mM KCl, 2 mM $MgCl_2$, 0.5% Nonidet P40, 0.5% Polyoxyethylene Sorbitan Monolaurate, and 40 μg/mL proteinase K) at 500 cells/μL overnight at 50 °C. After treatment at 95 °C for 10 min and serial 5-fold dilution, 2 μL of samples were used as template for PCR. Primers and their sequence used in each reaction were as follows: D–J rearranged IgH; DHL-5′ (GGAATTCG(A/C)TTTTTGT(C/G)AAGGGATCTACTACTGTG) and J3-3′ (GTCTAGATTCTCACAAGAGTCCGATAGACCCTGG), germline IgH; Mu0-5′ (CCGCATGCCAAGGCTAGCCTGAAAGATTACC) and J3-3, α-actin; actin-S (GGTGTCATGGTAGGTATGGGT) and actin-AS (CGCACAATCTCACGTTCAG).

2.9. RT-PCR

Total RNA was extracted from sorted fetal liver cells or non-adherent cells from cultures using the RNeasy mini RNA isolation kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. cDNA was synthesized from extracted RNA using oligo dT primers and Superscript III reverse transcriptase (Invitrogen). The amount of cDNA was normalized according to the amount of cDNA of hypoxanthine-guaninephosphoribosyltransferase (HPRT) and used as template in each reaction. Sequences of primers were as follows: EBF-5′, GCCTTCTAACCTGCGGAAATC; EBF-3′, GGCGCACATAGAAATCCTGTT; Pax5-5′, CTACAGGCTCCGTGACGCAG; Pax5-3′, TCTCGGCCTGTGACAATAGG; IL-5Rα-5′, ACATTTATCAACAGCAAAGGGTTT; IL-5Rα-3′, AGTTAAAGCAAT-