Table 1 Primers used for the amplifications and sequencing of MHC class I cDNA from cynomolgus monkeys

Primers	Binding region (position)	Sequence[a]
Primers for amplification		
5′ Beta 3 XMO[b]	All loci exon 1 (–27-1)	5′-CGC TCG AGG ACT CAG AAT CTC CCC AGA CGC CGA G-3′
Mafa-B1a	*B* and I loci exon 8 (1089–1117)	5′-CCA CTT AAG ACA GTT TCA GGC TTT T-3′
5′ MBS[b]	*B* and I loci exon 1 (10–34)	5′-GCC TCG AGA ATT CAT GGC GCC CCG AAC CCT CCT CCT GC-3′
3′ MBS[b]	*B* and I loci exon 8 (1095–1116)	5′-GCA AGC TTC TAG ACC ACA CAA GAC AGT TGT CTC AG-3′
Primers for sequencing		
T7 primer	pCR4Blunt-TOPO vector (328–347)	5′-TAA TAC GAC TCA CTA TAG GG-3′
T3 primer	pCR4Blunt-TOPO vector (243–262)	5′-AAT TAA CCC TCA CTA AAG-3′
Ia698	All loci exon 4 (680–698)	5′-TAG AAG CCC AGG GCC CAG C-3′
Is437	All loci exon 3 (437–456)	5′-ATT ACA TCG CCC TGA ACG AG-3′

[a]*Xho*I, *Sal*I, and *Hin*dIII sites of 5′ beta 3 XHO, 5′ MBS, and 3′ MBS primers, respectively, are *underlined*
[b]From Boyson et al. (1996b)

locus by Boyson et al. (1996b) were also used to amplify the cynomolgus MHC class I *B* locus. PCR amplification was performed at least twice for each animal. PCR products were cloned into pCR4Blunt-TOPO plasmids (Invitrogen, Carlsbad, Calif., USA) and 48 clones were sequenced by 310 Capillary DNA Sequencer (Applied Biosystems, Foster City, Calif., USA) or 3100-Avant Capillary DNA Sequencer (Applied Biosystems). The *Mafa-B* nucleotide sequences were assembled with the Contig Manager of the DNASIS pro (Hitachi Software, Yokohama, Japan). The Clustal W algorithm provided in DNASIS PRO was used to align sequences.

Phylogenetic analysis

The full-length nucleotide sequences of *Mafa-B*, *Mafa-I*, *Mafa-A*, *Mamu-A*, *Mamu-B*, *Mamu-I*, *HLA-A*, and *HLA-B* were aligned using Clustal W provided online by the DNA Data Bank of Japan [(DDBJ) http://www.ddbj.nig.ac.jp]. A phylogenetic tree of these nucleotide sequences was constructed by the neighbor-joining method of the Molecular Evolution Genetics Analysis, version 2.1 (MEGA 2.1). Genetic distances were estimated using the method of Jules-Canter. At the sites in which alignment indicated a gap, nucleotides at this position in all the sequences were deleted. The reliability of the tree topology was tested by the bootstrap method. Thousand relationships and 64,238 random seeds were used for determining bootstrap values (Fig. 2a, b). Since the bootstrap values of less than 50% were unreliable, the values of less than 50% were not shown in Fig. 2a, b.

	Clone /animal	Leader peptide -20 -10
HLA-B*2702		MRVT APRTLLLLLW GAVALTETWA
HLA-B*5701		....V..... .....
Mamu-B*02		...M .....S ..L.....
Mamu-B*03		...M ....F....S ..L.....
Mamu-B*04		...M ....F....S ..L.....
Mafa-B*01	7/2	...M .....S ..L.....
Mafa-B*02	3/1	.Q.M .....S ..L.....
Mafa-B*03	13/3	...M .....S ..L.....
Mafa-B*04	9/2	...M .....S .TLS.....
Mafa-B*05	36/2	...M ...I.....S .TLS.....
Mafa-B*06	6/1	...M ...I.....S .TLS.....
Mafa-B*07	28/1	...M .....S ..L.....
Mafa-B*08	2/1	...M .....S .TLV.....
Mafa-B*09	21/2	...M .....S ..L.....
Mafa-B*10	4/1	---- --------.S ..L.....
Mafa-B*11	16/1	---- --------.S ..L.....
Mafa-B*12	17/2	...M .....S ..L.....
Mafa-B*13	4/1	...M .....S ..L.....
Mafa-B*14	33/2	...M .....S .PL.....
Mafa-B*15	10/1	---- --------.S ..L.....
Mafa-B*16	25/4	...M .....S A.L...K...
Mafa-B*17	45/3	.Q.M .....S ..LS.....
Mafa-B*18	3/1	...M .....S ..L...Q...
Mafa-B*19	20/2	.QIM .....S ..L.....
Mafa-B*20	6/2	..DM .....S ..L.....
Mafa-B*21	22/2	.QIM .....S ..L.....
Mafa-B*22	36/2	...M .....S ..L.....
Mafa-B*23	22/2	...M .....S ..L.....R.
Mafa-B*24	10/3	...M ..G.....S ..L.....
Mafa-B*25	7/3	..FM .....S ..L...Q...
Mafa-B*26	5/1	...M ..G.....S ..L.....
Mamu-I*04		---- --------HS ..L.....
Mamu-I*07		--.M ..G.....S ..L.....
Mamu-I*08		---- --------.S ..L.....
Mafa-I*01013	13/1	...M .....S ..L.....
Mafa-I*02	12/1	...M ..G.....S ..L.....
Mafa-I*03	33/3	...M ..G.....S .TL.....
Mafa-I*04	2/1	...M ..G.....S ..L.....
Mafa-I*05	11/1	...M ..G.....S ..L.....
Mafa-I*06	16/2	...M ..G.....S ..L.....
Mafa-I*07	3/1	...M ..G.....S ..L.....
Mafa-I*08	5/1	...M ..G.....S ..L.....
Mafa-I*09	51/3	...M ..G.....S ..L.....
HLA-A*0201		.A.M .....V...S ..L...Q...
Mamu-A*01		---- .....V.S ..LV..Q.R.
Mamu-A*02		---- ----...V.S ..L...Q.R.
Mafa-A*01		.A.M .....V.S ..L...Q.R.
Mafa-A*02		.A.M .....V.S .VL...Q.R.
Mafa-A*06		.A.M .....V.S .FL...Q.L.

Fig. 1 Deduced amino acid sequences of *Mafa-B* and *Mafa-I* alleles. ▶ Amino acid sequences of *HLA-A*, *HLA-B*, *Mamu-A*, *Mamu-B*, *Mamu-I*, *Mafa-A*, and *Mafa-E* alleles were also included. Amino acids identical to those of *HLA-B*2702* are indicated by *dots*. The deletions of amino acids are indicated by *hyphens*. The total numbers of clones obtained and the numbers of animals having the allele were indicated *after the allele name*

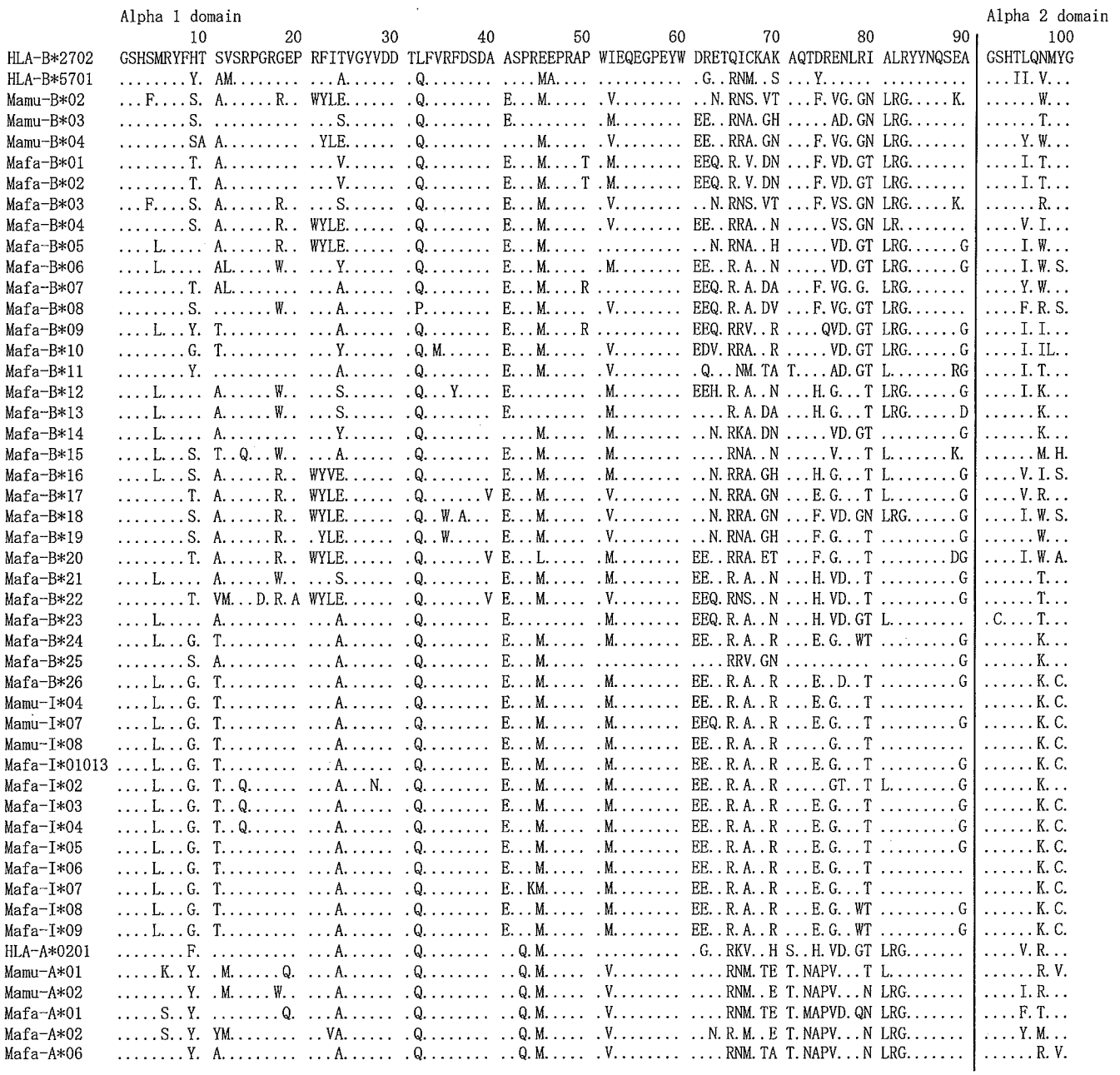

Fig. 1 (continued)

GenBank accession numbers

The *Mafa-B* and *Mafa-I* sequences described in this manuscript had been deposited in the DDBJ and were assigned accession numbers AB195431 to AB195465. We previously deposited *Mafa-A* alleles in the DDBJ, and these alleles were assigned accession numbers AB154760 to AB154773. The GenBank accession numbers for other sequences used in this study are as follows: *HLA-A*0201*, U07161; *HLA-B*2702*, L38504; *HLA-B*5701*, AJ458991; *Mafa-E*01*, U02976; *Mamu-A*01*, U50836; *Mamu-A*02*, U50837; *Mamu-A*03*, U41379; *Mamu-A*04*, U41380; *Mamu-B*02*, U41833; *Mamu-B*03*, U41825; *Mamu-B*04*, U41826; *Mamu-B*05*, U41827; *Mamu-B*06*, U41828; *Mamu-B*07*, U41829; *Mamu-B*08*, U41830; *Mamu-B*36*, AJ556886; *Mamu-I*01011*, AF161865; *Mamu-I*02012*, AF161869, *Mamu-I*04*, AF4161874; *Mamu-I*07*, AF161875; *Mamu-I*08*, AF161876; *Mamu-I*09*, AF161877; *Mamu-I*10*, AF161878; and *Mamu-I*11*, AF161879.

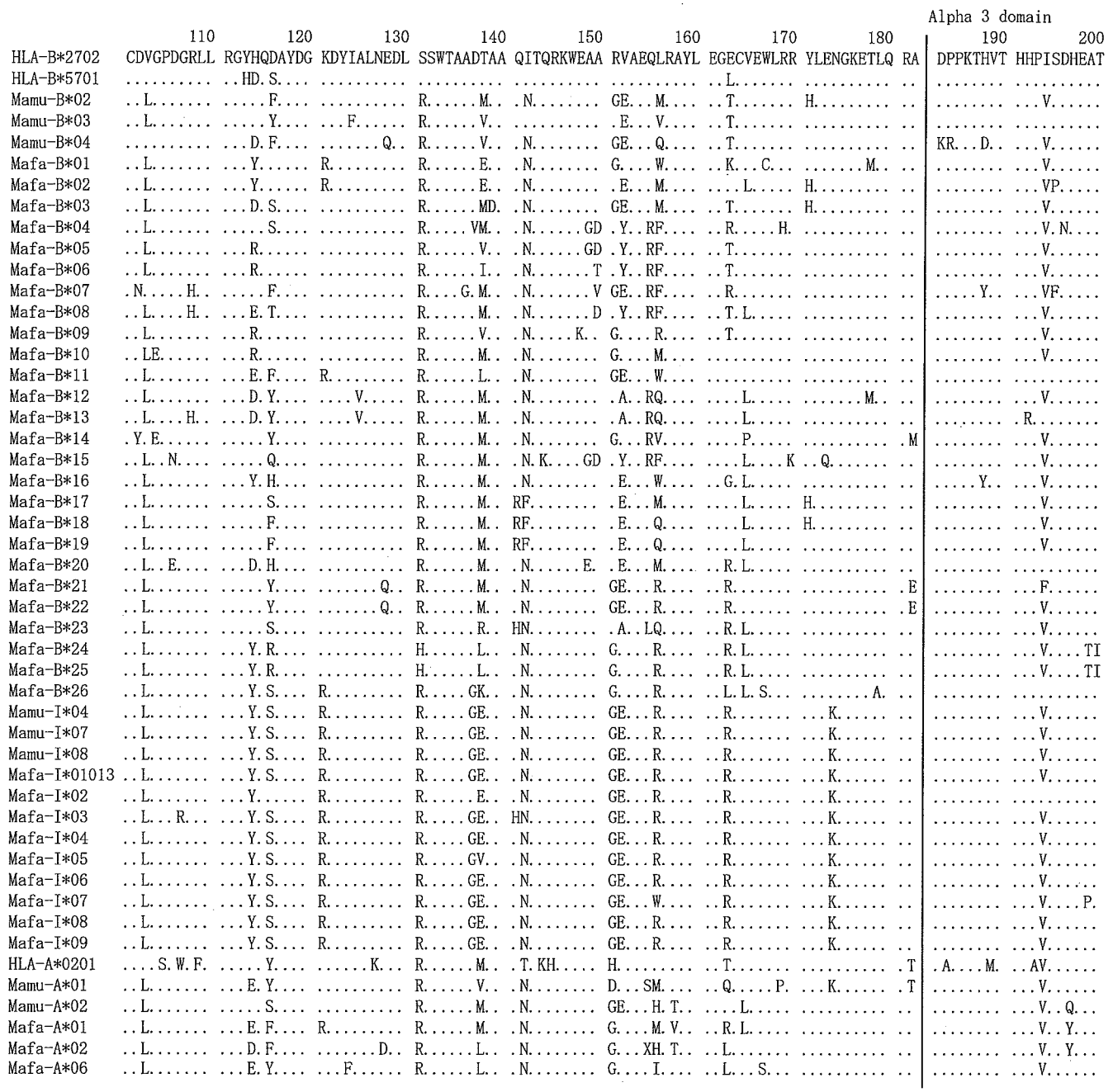

Fig. 1 (continued)

Results

Detection of 26 MHC class I *B* locus alleles and nine *I* locus alleles in cynomolgus monkeys

To amplify cynomolgus MHC class I *B* locus genes, PCR was carried out using primers that were successfully used for amplification of rhesus MHC class I *B* locus genes along with newly designed ones (Table 1; Boyson et al. 1996b). We obtained 48 clones from each animal. The nucleotide sequences that were found in just one clone were excluded from the subsequent analyses to avoid incorporation of artificial sequences generated by PCR error or during the cloning procedure into public databases. Ambiguous sequences were also excluded. When the nucleotide sequence was shared by more than two clones, regardless of whether they were derived from one animal or multiple animals, the sequences were regarded as a consensus sequences representing a particular alleles of each animal. Eventually, 43 candidate alleles were obtained, and 34 of 43 were found to have substantial homology with *Mamu-B* alleles. Amino acid sequences deduced from the nucleo-

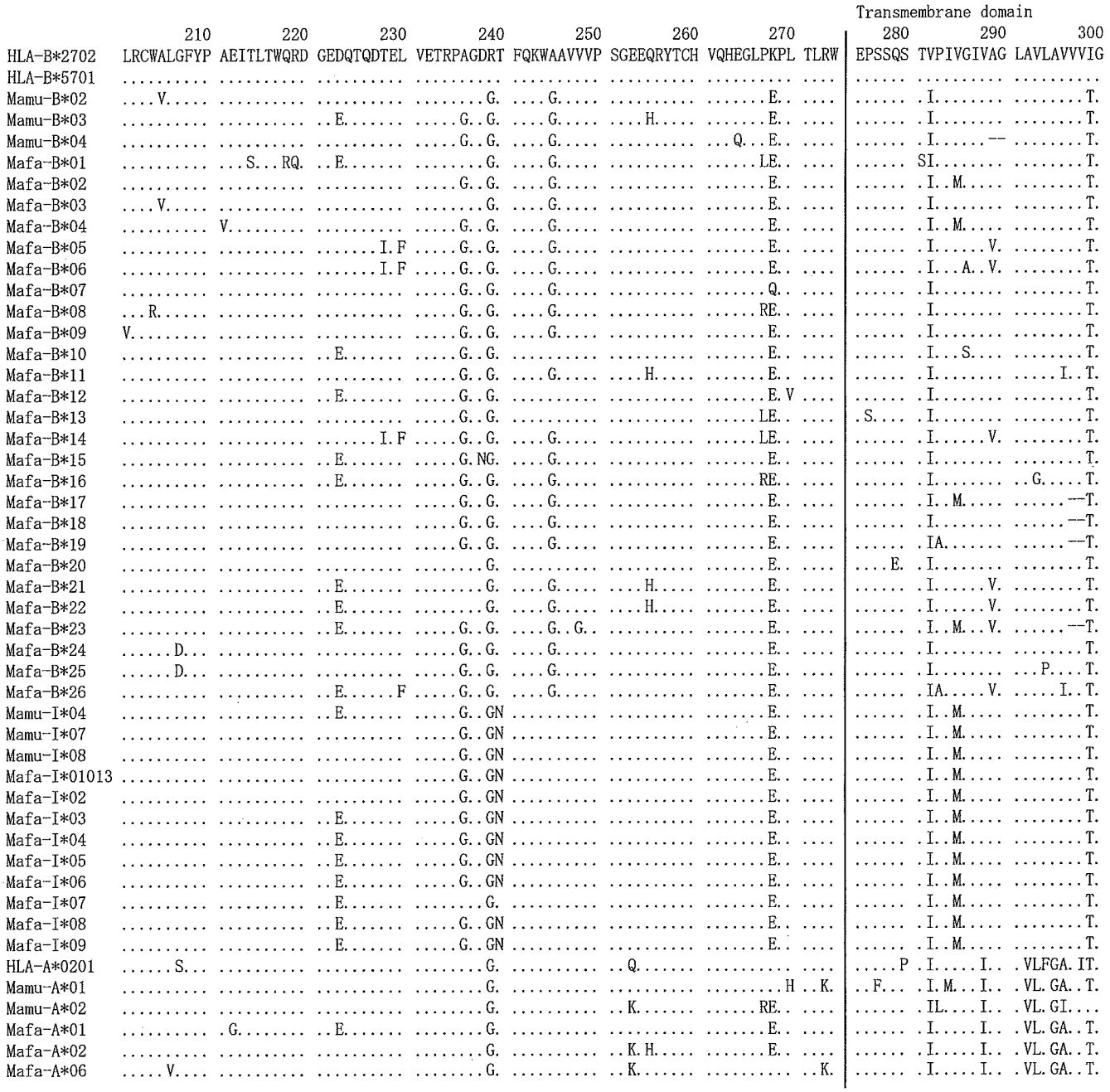

Fig. 1 (continued)

tide sequences of these 34 candidate *B* alleles were further subjected to phylogenetic analysis using the neighbor-joining method (Saitou and Nei 1987; data not shown). When the predicted amino acid sequence variation between two candidates was negligible (d<0.025), the amino acid sequence shared by a majority of the clones was regarded as representing a particular allele. The other sequence shared by a minority of clones was excluded from the subsequent analyses. As the result of the analysis, 26 *Mafa-B* alleles were identified. It was found that the remaining nine candidate alleles were closely related to those of *Mamu-I* locus reported by Urvater et al. (2000b). Since Urvater et al. also identified two *Mafa-I* alleles (*Mafa-I*01011* and *Mafa-I*01012*), we named tentatively alleles identified here *Mafa-I*01013* through *Mafa-I*09*. The *Mafa-I*01013* allele was identical in amino acid sequence with *Mafa-I*01011* and *Mafa-I*01012*, but there were several synonymous nucleotide changes scattered around the sequence. We therefore considered that this alleles was a variant of *Mafa-I*01*, although reported sequences of *Mafa-I*01011* and *Mafa-I*01012* were incomplete. The deduced amino acid sequences of *Mafa-B* and *Mafa-I* alleles were shown in Fig. 1

```
                                  Cytoplasmic domain
                          310         320         330         340
HLA-B*2702    AVVAAVMC  RR KSSGGKGGSY SQAACSDSAQ GSDVSLTA*- --
HLA-B*5701    ........  .. .......... .......... .......... -- --
Mamu-B*02     .......W  .. .......... ....S..... .......... -- --
Mamu-B*03     .......W  .. .......... ....SN.... .......... -- --
Mamu-B*04     .......W  .. .......... ....S..... .......... -- --
Mafa-B*01     .......R  .. .......... ....S..... .......... -- --
Mafa-B*02     .....M..  .K .T........ F...SK.... ......M.. -- --
Mafa-B*03     .......W  .. .......... ....S..... .......... -- --
Mafa-B*04     .......W  .. .......... ....S.N... .......... -- --
Mafa-B*05     .......W  .. .......... ....SN.... .......... -- --
Mafa-B*06     .......W  .. .......... ....SN.... .......... -- --
Mafa-B*07     .......W  .. .......... ....S..... .......... -- --
Mafa-B*08     .......W  .. .......... ....S..... .......... -- --
Mafa-B*09     .......W  .. .......... ....SN.... .......... -- --
Mafa-B*10     .......W  .. .......... ....S..... .......... -- --
Mafa-B*11     .......W  .. .......... ....S..... .......... -- --
Mafa-B*12     .......W  .. .......... ....S..... .......... -- --
Mafa-B*13     .......W  .. .......... ....S..... .......... -- --
Mafa-B*14     .......W  .. .......... ....SN.... .......... -- --
Mafa-B*15     .......W  .. .......... ....SN.... .......... -- --
Mafa-B*16     .......W  .K ...S..R.. .... S..... ......M.. -- --
Mafa-B*17     .....M..  .. .T.....R.. F...SK..P. ...E..M.. -- --
Mafa-B*18     .....M.R  .. .......... F...SK..P. ...E..RS. -- --
Mafa-B*19     .......W  .. .......... F...SK..P. ...E..M.. -- --
Mafa-B*20     .......W  .. .......... ....S..... .......... -- --
Mafa-B*21     .......W  .. .......... ....S..... .......... -- --
Mafa-B*22     .......W  .. .......... ....S..... .......... -- --
Mafa-B*23     .......W  .K RT...R.... F...S..... .......... -- --
Mafa-B*24     .......W  .. .......... ....S..... .......... -- --
Mafa-B*25     .......W  .. .......... ....S..... .......... -- --
Mafa-B*26     .......W  .. .......... ....SN.... .......... -- --
Mamu-I*04     .......W  .. .......... ....S..... .......... -- --
Mamu-I*07     .......W  .. .......... ....S..... ..........-- --
Mamu-I*08     .......W  .. .......... ....S.N... .......... -- --
Mafa-I*01013  P......W  .. .......... ....S.N... .......... -- --
Mafa-I*02     P......W  .. .......... ....S.N... .......... -- --
Mafa-I*03     .......W  .. .......... ....S.N... .......... -- --
Mafa-I*04     .......W  .. .......... ....S.N... .......... -- --
Mafa-I*05     .......W  .. .......... ....S.N... .......... -- --
Mafa-I*06     .......W  .. .......... ....S..... .......... -- --
Mafa-I*07     .......W  .. .......... ....S.N... .......... -- --
Mafa-I*08     .......W  .. .......... ....S..... .......... -- --
Mafa-I*09     .......W  .. .......... ....S.N... .......... -- --
HLA-A*0201    .......W  .. ...DR..... ....S..... ........CK V*
Mamu-A*01     .......W  .. ...DR..... ....S..... ........CK V*
Mamu-A*02     ..I...IW  .. ...DR..... ....S..... ........CK V*
Mafa-A*01     .......W  .. ...DR..... ....SN.... ........CK V*
Mafa-A*02     ....V..W  .. ...DR..... ....S..... ........CK V*
Mafa-A*06     ...T...W  .. ...DR..... ....S..... ........CK V*
```

Fig. 1 (continued)

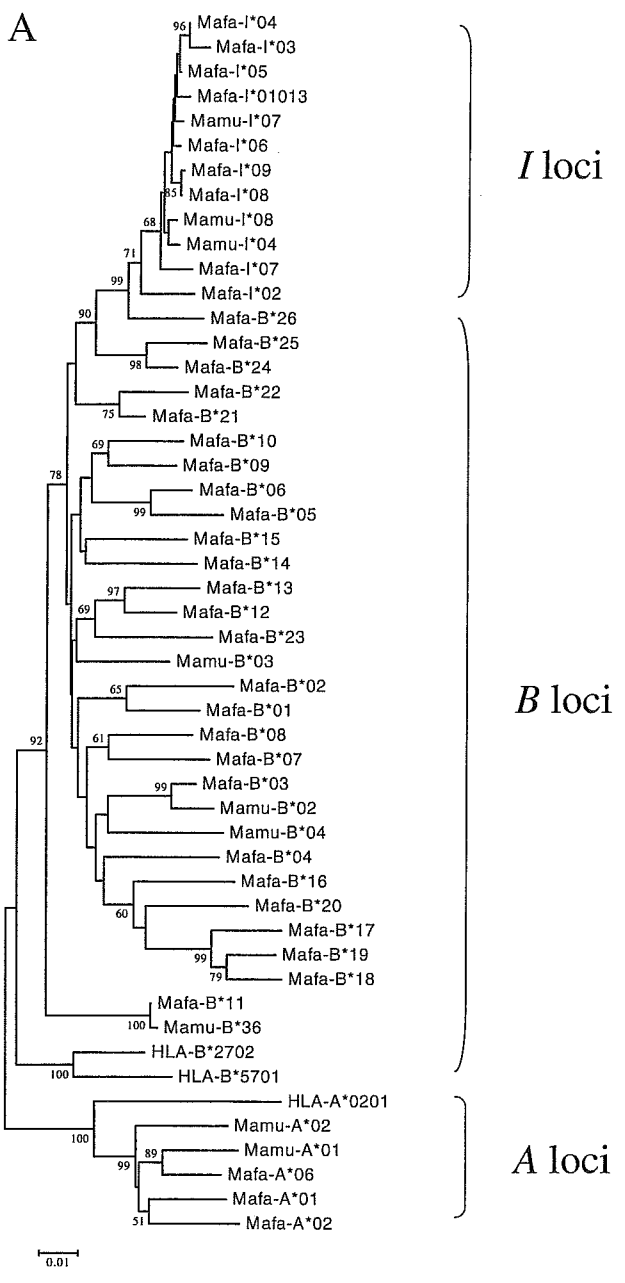

Fig. 2 Phylogenetic analysis of primate class I MHC molecules. The phylogenetic tree was constructed using **a** full-length and **b** exon five to eight nucleotide sequences by neighbor-joining method with MEGA2.1. The bootstrap values of more than 50% were shown

along with those of alleles reported for other primates. The total numbers of clones obtained and the numbers of animals having the allele were shown in the figure. The putative glycosylation site was located at residue 86, and the conserved cysteine residues occurred at positions 101 and 164 in $\alpha 2$ and at positions 203 and 259 in $\alpha 3$. To evaluate whether the nucleotide sequences of *Mafa-B* and *Mafa-I* alleles established in this study were gene products of class I *B* and *I* loci, respectively, *Mafa-B* and *Mafa-I* alleles were phylogenetically analyzed (Fig. 2a). The full-length nucleotide sequences of *Mafa-B*, *Mafa-I*, *Mafa-A*, *Mamu-A*, *Mau-B*, *Mamu-I*, *HLA-A*, and *HAL-B* were aligned by Clustal W. A phylogenetic tree was constructed by the neighbor-joining method of MEGA2.1 software. The reliability of the tree topology was tested by the bootstrap method, and the bootstrap values are shown in Fig. 2a. Since the bootstrap values of less than 50% were unreliable, the bootstrap values of greater than 50% are shown in Fig. 2a. Several *Mafa-B* alleles (*Mafa-B*21*, *22*, *24*, *25*, and *26*) appeared to cluster with *Mamu-I* or *Mafa-I* allele rather than *B* locus alleles. Since amino acid difference between alleles of *I* and *B* loci were more apparent at the carboxy half of the protein, we recon-

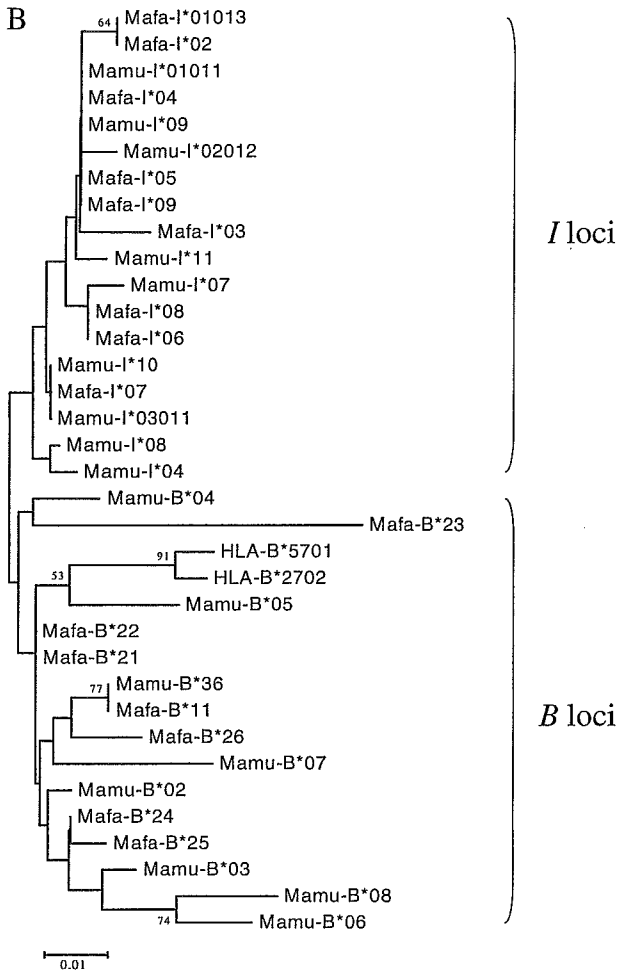

Fig. 2 (continued)

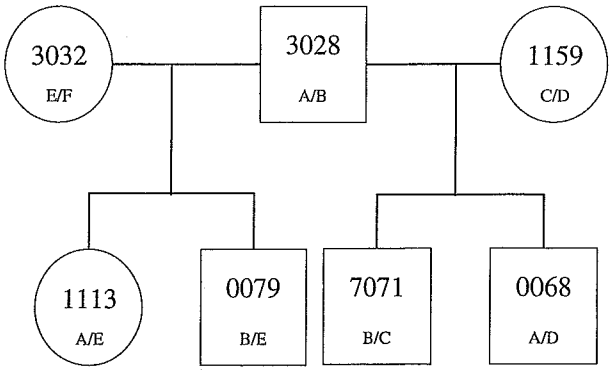

Fig. 3 The family pedigree demonstrating the inheritance of alleles of the MHC class I *B* and *I* loci of cynomolgus monkeys. Male and female are denoted by *squares* and *circles*, respectively. The animal number assigned to the animal is shown. The haplotypes of each animal are given by *capital letters*

structed the phylogenetic tree using the amino acid sequences of the exons 5 to 8. The result clearly showed that these nine alleles clustered with *Mamu-I* alleles (Fig. 2b). These results strongly suggested that these cDNA clones were derived from distinct alleles on MHC class I *B* and *I* loci of cynomolgus monkeys.

Inheritance of *Mafa-B* and *Mafa-I* in a family of cynomolgus monkeys

A family consisting of three parents (one sire and two dams) and four offspring was subjected to genetic analysis to study inheritance of *Mafa-B* and *Mafa-I* alleles. By nucleotide sequence analysis, ten *Mafa-B* alleles and four *Mafa-I* alleles were detected in this family as shown in Table 2. Since certain alleles appeared to be inherited in this family as a complex, we considered those gene complexes as haplotypes and assigned letters A through F to those combinations of alleles (Table 2). Haplotype A (*Mafa-B*03* and *Mafa-I*09*)

Table 2 Segregation of alleles with haplotypes

Haplotypes	Alleles	Animal no.						
		3032	3028	1159	1113	0079	7071	0068
A	*Mafa-B*03*		○		○			○
	*Mafa-I*09*		○		○			○
B	*Mafa-B*24*		○			○	○	
	*Mafa-B*25*		○			○	○	
C	*Mafa-B*17*			○			○	
	*Mafa-B*20*			○			○	
	*Mafa-I*06*			○			○	
D	*Mafa-B*01*			○			○	
	*Mafa-B*04*			○				○
E	*Mafa-B*16*	○			○	○		
	*Mafa-I*03*	○			○	○		
F	*Mafa-B*06*	○						
	*Mafa-B*23*	○						
	*Mafa-I*02*	○						

○ :Alleles were detected in each individual

Table 3 The presence of multiple *Mafa-B* alleles in cynomolgus monkeys

Animal no.	Alleles	Number of copies	Primers
2010	*Mafa-B*09*	5	5′MBS/3′MBS, 5′Beta 3 XHO/Mafa-Bla
	*Mafa-B*11*	16	5′MBS/3′MBS, 5′Beta 3 XHO/Mafa-Bla
	*Mafa-B*12*	7	5′MBS/3′MBS, 5′Beta 3 XHO/Mafa-Bla
	*Mafa-B*19*	2	5′MBS/3′MBS, 5′Beta 3 XHO/Mafa-Bla
5076	*Mafa-B*10*	4	5′MBS/3′MBS
	*Mafa-B*14*	17	5′MBS/3′MBS, 5′Beta 3 XHO/Mafa-Bla
	*Mafa-B*15*	10	5′MBS/3′MBS
	*Mafa-I*010103*	13	5′MBS/3′MBS, 5′Beta 3 XHO/Mafa-Bla

was detected in 3028, 1113, and 0068, whereas haplotype B (*Mafa-B*24* and *Mafa-B*25*) was carried by 3028, 0079, and 7071 (Fig. 3). Haplotype C (*Mafa-B*17*, *Mafa-B*20*, and *Mafa-I*06*) was found in 1159 and 7071, haplotype D (*Mafa-B*01* and *Mafa-B*04*) in 1159 and 0068, haplotype E (*Mafa-B*16*, *Mafa-I*03*) in 3032, 0079, and 1113, and haplotype F (*Mafa-B*06*, *Mafa-B*23*, and *Mafa-I*02*) in 3032 (Fig. 3). We could not detect *Mafa-I* alleles in monkeys bearing haplotypes B and D. It was evident that *Mafa-B* alleles were inherited in a Mendelian fashion. Moreover, cynomolgus monkeys in this family were shown to have two to four *Mafa-B* alleles. The presence of multiple *Mafa-B* alleles was confirmed by nucleotide sequences analysis of two additional cynomolgus monkeys unrelated to this family. Table 3 showed that 2010 had four *Mafa-B* alleles and 5076 had three *Mafa-B* alleles. These results indicated that MHC class I *B* locus of cynomolgus monkeys was duplicated as in the case of rhesus monkeys (Boyson et al. 1996b).

Discussion

Although cynomolgus monkeys are widely used as animal models in a variety of biomedical researches, there are no nucleotide sequence data on cynomolgus MHC class I *B* locus. In this study, we tried to identify the alleles of cynomolgus MHC class I *B* locus, using PBMC cDNA from 16 cynomolgus monkeys.

Nucleotide sequence analyses and following phylogenetic analysis identified 26 *Mafa-B* alleles (Figs. 1, 2a). We also found nine clones with the nucleotide sequences showing high homology with those of *Mamu-I* alleles. Phylogenetic analysis showed that these clones were derived from nine *Mafa-I* alleles. It was reported that novel MHC class I *I* locus in rhesus monkeys, *Mamu-I*, could be amplified with *B* locus-specific primers, and that the *I* locus was recently evolved from a classical MHC class I *B* locus by duplication (Urvater et al. 2000b).

The haplotypes of rhesus MHC class I composed of at least one *A* locus and at least two *B* loci (Boyson et al. 1996b). In cynomolgus monkeys, we previously reported that the *A* locus had been duplicated, because one to four *Mafa-A* alleles were found in an animal (Uda et al. 2004). The presence of up to six *Mamu-B* alleles in a rhesus monkey (Urvater et al. 2000a) indicates that rhesus monkeys have three class I *B* loci. In this study, we also showed that two to four *Mafa-B* alleles were present in each individual, strongly suggesting that cynomolgus monkeys have multiple MHC class I *B* loci. Regarding the *I* locus, it seemed possible that at least one locus was present in each animal, although some individual appeared not to have the locus. The apparent lack of the *I* locus in some individual was probably due to low efficiency of amplification of the *I* locus because of the presence of the multiple *B* loci.

Information on MHC class I molecule is particularly important in better understanding of pathogenesis of various infectious diseases including HIV infection. So far the nucleotide sequence data are available for the alleles of *Mafa-A* (Uda et al. 2004), *Mafa-E* (Alvarez et al. 1997; Boyson et al. 1995), *Mafa-G* (Arnaiz-Villena et al. 1997; Castro et al. 1996), *Mafa-I* (Urvater et al. 2000b), *Mafa-DRB* (Gaur et al. 1997; Kriener et al. 2000; Leuchte et al. 2004), *Mafa-DQA* (Kenter et al. 1992), and *Mafa-DQB* (Otting et al. 2002) in cynomolgus monkeys. The identification of *Mafa-B* alleles would, therefore, greatly help understand the pathogenesis of various pathogens that naturally or experimentally infect cynomolgus monkeys.

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実験用霊長類の心理的ストレスを評価する免疫学的指標と飼育環境のエンリッチメント評価

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Immunological markers for evaluation of the stress in laboratory primates and assessment of environment enrichment.

Keiji TERAO, Takamasa KOYAMA and Juri SUZUKI

Abstract　During the past two decades, a new research area has been developed to clarify the interaction or relationship between immune system and central nerves system (CNS). This scientific field needs interdisciplinary research and is designated as "Psychoneuro-immunology" or "Neuroimmunomodulation". Both the CNS and the immune system play an important role in maintaining normal physiological equilibrium or homeostatis and there are close interactions between them. The resent scientific interest in the relations between the CNS and the immune system focuses on the effects of psychological or psychosocial stress on immune functions in human. The relationship between bereavement and increase of mortality is well-known. However, given the methodological difficulties in quantifying stress and in it's induction in humans, a appropriate models is necessary to demonstrate how psychological factors are translated into pathological change in the immune system. Nonhuman primates are thought to be the most suitable models in this research area, because they are the only species that show the same behavioral and endocrine response to social or psychological stress as human. For establishing nonhuman primate model in stress-immune study, the following problems must be previously clarified. That is [1] which procedure is the most effective in stress induction? and [2] which immune function is mostly affected by psychological stress?

The most important matter in the case of evaluation of well-being status is how to select the suitable parameter for assessment. We have been making efforts to establish the standard procedures of care and management to keep laboratory primates under well-being conditions. The standardized procedure assures the reliable and reproducible results from animal experiment since it has been well known that laboratory environment must affect the psychological and physiological condition of animals. The process of improvement of standard management procedure must be the process of establishment of well-being condition in laboratory animals. The current situation regarding nonhuman primates in biomedical research offers the promise of future progress under refined procedures and facilities.

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はじめに

最近，気の持ち方や生き甲斐などの心理的・精神的要因がガンや成人病などの発症や治癒に大きく関わっていることを示す一般向け医療解説書が人気を呼んで

いるが，これらは「病は気から」の経験則を科学的に実証したここ10数年の研究成果が背景となっている。実験動物を含む大半の動物は変化する外部環境に対応して体内環境を一定に維持するための恒常性維持機構を備えている。生体の恒常性を維持するために様々な機構が存在するが，中でも神経系，内分泌系，免疫系は全身に機能的ネットワークを形成しているとともに，三者の密接な相互作用が恒常性維持に重要な役割を果たしていることが明らかになってきた。神経・内分泌・免疫相関(クロス・トーク)である。なかでも，恒常性の維持を外界からの防御機構としてとらえた場合には，神経系と免疫系のネットワークは最も重要なシステムとなる。しかしながら，神経系，免疫系ともにそれぞれが非常に複雑なシステムであり，両者の相互作用を科学的に解明するためには，優れた実験モデルや実験系の開発が必須である。恒常性維持に関わる二つのシステムの相互作用を解析する方法としては，恒常性を攪乱することにより変化する因子を明らかにする戦略が合理的である。神経・免疫相関を攪乱する要因として最もよく知られているのが心理的，社会的ストレスである。ストレスという言葉は複雑化する現代社会を象徴するキーワードとして日常的に用いられているが，「ストレス」を生体の恒常性維持に有害な刺激(ストレッサー)または有害刺激に対する生体反応(ストレス反応)と定義すれば，心理的，社会的ストレスの負荷実験は神経・免疫相関を解析する有用な実験系となる。我々は，実験用サル類の特性を利用してこれまで心理的，社会的ストレス負荷に伴う行動，内分泌，免疫系の変化を解析してきた。サル類をモデルとした神経・免疫相関解析についてはすでに報告しているので，本稿ではこれまでの成果を概説し，心理的，社会的ストレス負荷にともない変化する免疫系，内分泌系，行動のそれぞれの指標を用いて実験用サル類の環境エンリッチメントまたは心理学的安寧を評価する試みを紹介する。

1．心理的，社会的ストレス環境における生体反応

集団での生活が中心となる多くのサル類ではヒトと同様に他個体との関係調節が個体にとって必須の社会的行為であり，近親個体との離別や未知の他個体との遭遇は，著しい心理的，社会的ストレスとなる。このようなサル類の心理，行動学的特性を神経，免疫相関の解析に応用する試みとして，母子分離，同胞分離，社会的隔離，群形成，群導入，社会的順位，優劣関係，社会的競合等の様々な手続きが試みられている。なかでも，母子分離はいずれのサル種においても仔ザルに典型的なストレス反応を生じさせ，分離直後には異常な興奮状態を反映するとみられる種々のステレオタイプ行動が多発し，続いて極端な抑鬱状態におちいる。図１は母子分離(離乳)後のカニクイザル仔ザルの行動(抑鬱行動)，内分泌系(コーチゾル)，免疫系(NK細胞，U５陽性細胞)の変化を要約したものである。生後５～

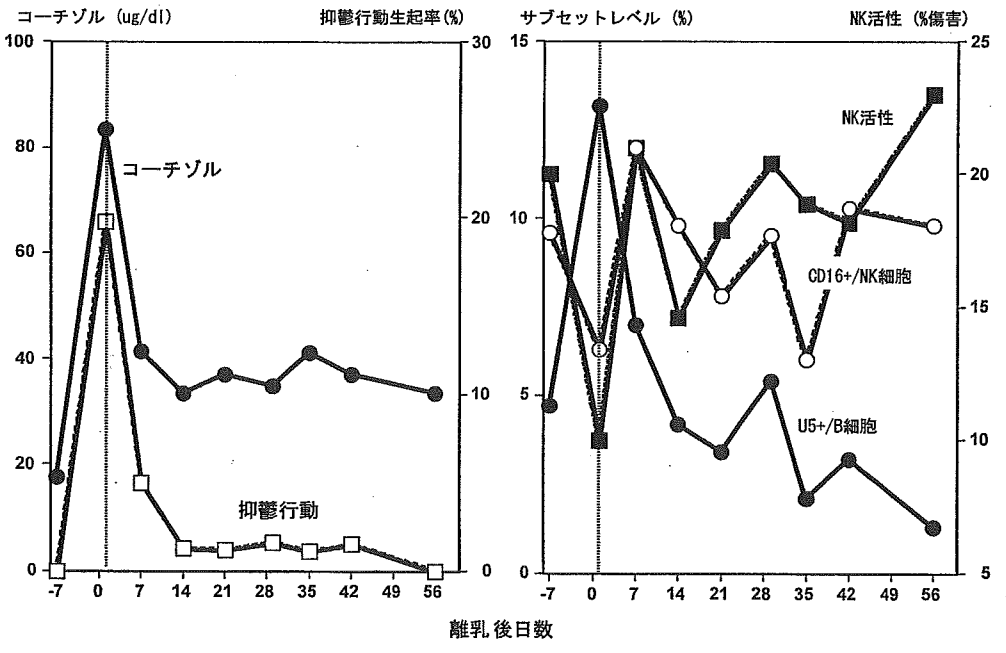

図１　離乳のストレスがカニクイザル仔ザルの行動，内分泌，免疫系に及ぼす影響

6ヶ月間実母により保育された仔ザルは，離乳後数頭の仔ザルと群飼育される。当然，離乳後1日目のストレスは大きく，抑鬱行動および血中コーチゾルレベルが著しく増加するとともに，CD16陽性のナチュラルキラー(NK)細胞が低下し，同時にNK活性が著しく低下する。これに対して，U5陽性のB細胞(U5+/B)レベルは著しく上昇する。詳述は避けるがU5+/B細胞は我々が新たに見いだしたストレス関連リンパ球であり，マカクザルおよびヒトで心理的ストレス負荷後に上昇することを明らかにしている。図1に示すようにすべての指標は分離後一日目に著しい変化を示すが，一週後にはほぼ離乳前の値に回復する。すなわち，カニクイザルにおいては母子分離のストレスは高レベルではあるが一過性のストレスであり，離乳仔ザルは一週間程度で新しい環境に順応する。図1に示す項目はいずれもカニクイザルのストレス反応を評価する有用な指標であることが明らかとなった。これらのストレス評価指標は神経・内分泌・免疫相関を解析するマーカーであると同時に，実験用霊長類の飼育管理において要求される環境エンリッチメントや心理的安寧の実態を評価する指標として用いることも可能である。そこで，これらの指標を用いて飼育下にあるカニクイザルのストレスを評価し，ストレスを指標としてカニクイザルの飼育形態のありかたを検討してみた。

2．複数飼育により実験用霊長類の飼育環境エンリッチメントと心理的安寧は保証されるか？

近年，欧米を中心にして動物実験における動物福祉，動物の権利に関する社会的関心が高まり，実験動物の飼育環境の適正化への対応が求められるようになってきた。特に大型で知能の発達しているサル類は実験動物の中でも特殊な動物であり，その飼育管理には特殊な施設，設備管理技術に加えて，動物福祉の立場から環境エンリッチメントや心理的安寧への配慮が要求される。欧米のガイドラインでは社会的動物であるサル類の行動特性に配慮して個別飼育を避け，複数での飼育環境が推奨されている。しかしながら，完全隔離されたサル類では異常行動や発達異常が生じることは周知の事実であるが，実験動物としてのサル類を管理する立場からすれば，集団飼育に比べ個別飼育の方が個体管理や感染症防御等の点で優れている。また，個体関係の調整という観点からは，限られたスペースでのケージ内飼育と野外での集団生活では著しく状況が異なっている。さらに，社会的動物であるサル類では集団飼育が推奨される一方で，個別飼育と集団飼育で心理的ストレスの影響を比較した報告は少ない。個別飼育に順応したカニクイザルを集団飼育した場合のストレスを評価することは，飼育環境下における集団飼育の是非を議論する一助ともなる。そこで，筑波霊長類センターで個別飼育されてきた20歳以上の老齢カニクイザルと4～5歳の若齢カニクイザルとを同一のケージに同居させ，同居後のストレスを評価することにより集団飼育の必要性を評価してみた。幅120cm，高さ60cm，奥行き60cmの連結ケージにそれぞれ個別ケージで飼育されていた老齢雌ザルと若齢雌ザルをそれぞれ一頭ずつ同居させ，同居前，同居直後，1日，7日，14日後に先に示したストレス評価指標(行動，コーチゾル，CD16+/NK細胞，U5+/B細胞)の変化を調査した。

図2は同居前後での6種類の行動の生起率を老齢ザル，若齢ザルそれぞれ8頭で調査した結果を平均値で示す。ここに示す行動の中では「接触行動」だけが個体間で生じる行動であるが，同居直後に若齢ザルから老齢ザルに対する「接触行動」が増加しているのがわかる。図3は個体間の関係調節に関連する3種類の行動(グルーミング，威嚇行動，恐れ/回避行動)の生起率を示したものである。図2でみられた同居直後の「接触行動」はその大半が若齢ザルから老齢ザルへの「グルーミング(毛繕い)」であることがわかる。一方，老齢ザルは同居日数の増加に伴い大変奇妙な行動を示す。同居直後に若齢ザルからの「グルーミング」を受けながらも「恐れ/回避行動」がみられ，同居後7日目には「威嚇行動」が現れる。これに反応して若齢ザルでは同居7日後に「恐れ/回避行動」が増加するが，老齢ザルに対する「グルーミング」はみられない。同居後14日では老齢ザルの「威嚇行動」がみられるものの，若齢ザルは「グルーミング」も「恐れ/回避行動」も示さない。逆に老齢ザルではふたたび「恐れ/回避行動」の生起率が増加する。同居14日目での老齢ザルは，若齢ザルを威嚇しながら恐れるという大変矛盾した(Ambivalent)な心理状態に陥ることが推測され，若齢ザルとの同居期間が長引くにつれて，次第に同居に伴う心理的負荷が増加してゆくことが示唆される。この状態を内分泌および免疫系の変化で見ると(図4)，老齢ザルが受けるストレスの大きさが反映されている。すなわち，若齢ザルでは同居後7日まではコーチゾルレベルが上昇するが，14日目には低下を始めている。NK細胞レベルも同居直後に一過性に上昇するが14日目には同居前のレベルまでに低下する。若齢ザルでは同居後14日で同居の慣れが生じているのかもしれない。これに対して，老齢ザ

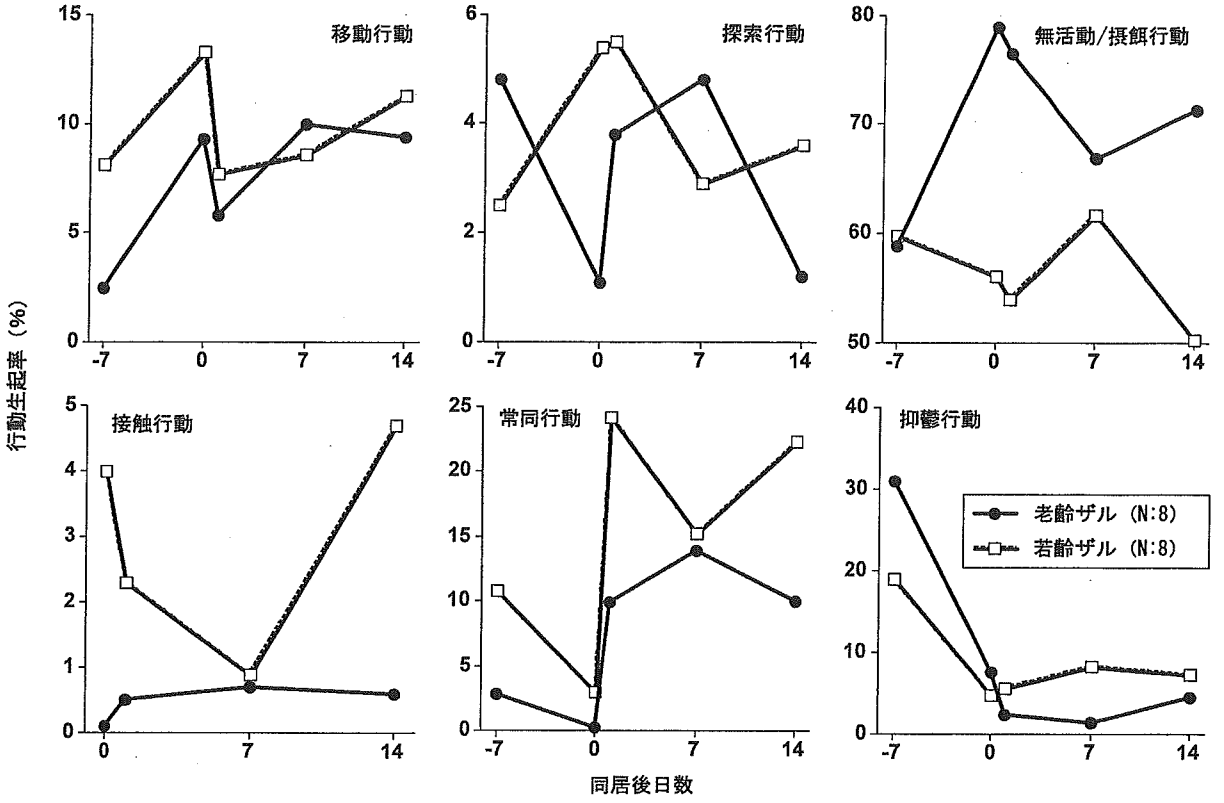

図２　同居のストレスが老齢ザルと若齢ザルの行動に及ぼす影響－１

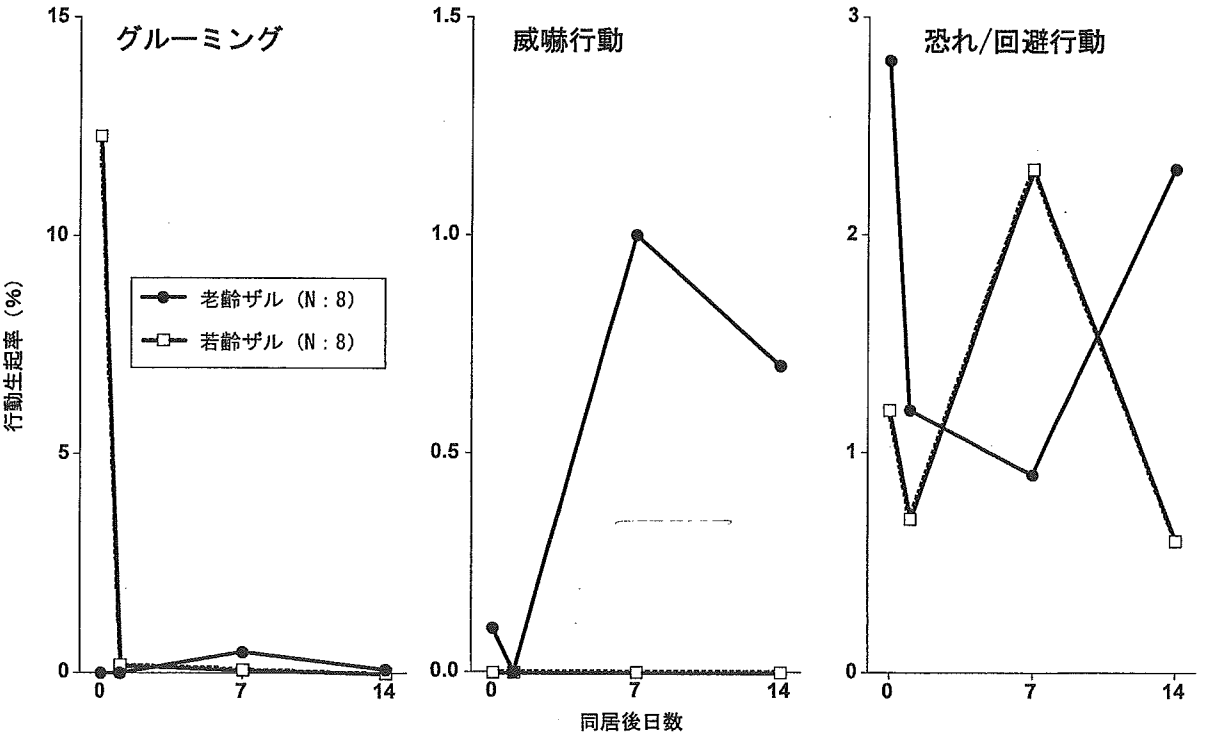

図３　同居のストレスが老齢ザルと若齢ザルの行動に及ぼす影響－２

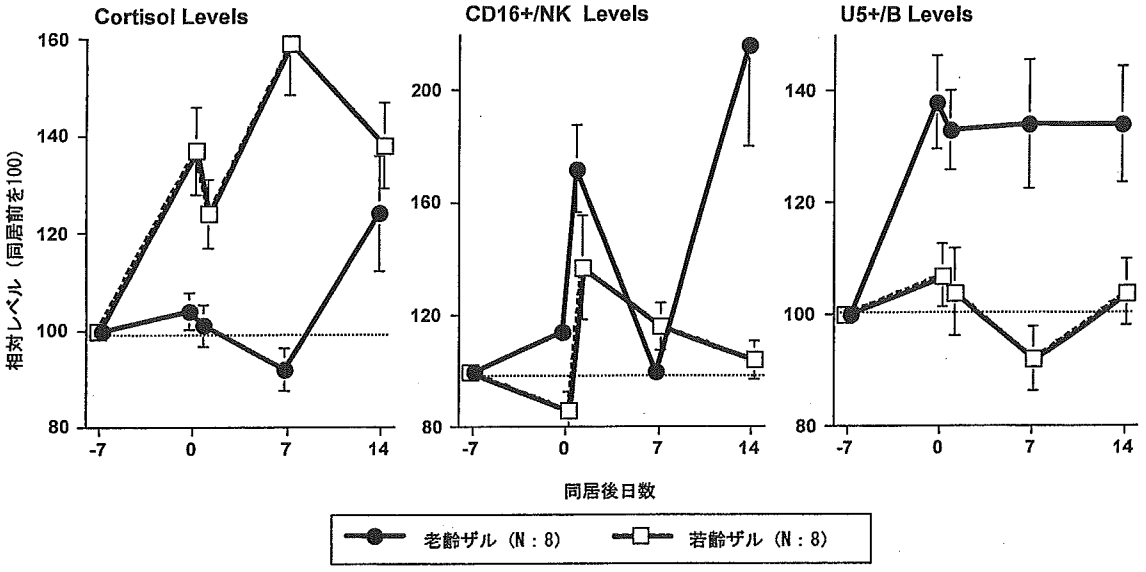

図４　同居のストレスが老齢ザルと若齢ザルのコーチゾルおよびリンパ球サブセットレベルに及ぼす影響

ルでは図３で示した同居後14日目のAmbivalentな心理状態を反映して，コーチゾルレベルもNK細胞レベルも急激に上昇している。これらの結果は，老齢ザルでは同居直後よりもむしろ同居期間が長引くにつれて個体関係の調整からくる心理的ストレスが増加していることを示唆している。興味あることは，老齢ザルの場合のみU５+/B細胞のレベルが同居直後に急増し同居期間中を通じて高いレベルを維持していることである。腎移植患者での調査結果からU５+/B細胞の増加は一過性のストレスよりも長期慢性ストレスを反映している可能性が考えられていたが，図４の結果から老齢ザルでは未知の他個体との同居が低レベルではあるが持続する慢性ストレスとなり，同居後に個体関係の調整に様々な努力をするものの，14日目に限界に近い状態になる可能性を示している。サル類が社会的動物であるが故に，突然の無差別な同居は特に高齢ザルにおいて持続する慢性ストレスとなる可能性が高い。ここに示す結果は，実験用サル類における飼育環境への環境エンリッチメントおよび心理的安寧の配慮では，無差別な集団飼育がもたらす負の影響について慎重な配慮が必要であることを示している。

３．心理的ストレスを評価する二つの免疫学的指標は同一の神経支配を受けているか？

これまでに，ヒトを含めた霊長類の心理的，社会的ストレスを評価する免疫学的指標としてCD16+/NK細胞とU５+/B細胞の二種類のマーカーが有用であることを示してきた。一般にストレスに対する反応は，間脳－下垂体－副腎皮質を介した系と，自律神経－副腎髄質を介した系が存在することが知られている。二つの免疫学的指標，CD16+/NK細胞とU５+/B細胞がどちらのシステムに支配されているかは興味ある課題であるが，サル類で実証するためには様々な侵襲的処置が必要となり，実験処置そのものがストレスとなる可能性が高い。直接的証明はともかく，少なくとも両者が同一のシステムに支配されているか否かを明らかにしておく必要がある。我々は非侵襲的方法により両者の関係を解析する方法として，日内変動に着目して解析を行った。ストレス実験で最も問題になるのは採血時のストレスをどう回避するかであるが，末梢リンパ球サブセットレベルの日内変動調査では，無拘束連続採血装置（チター装置）による採血が必要である。図５は，７頭のアカゲザルと４頭のニホンザル，計11頭にチター装置を装着し，午後４時（16：00）から翌日の午後４時までの24時間で４時間置きに７回採血した。照明は午後７時消灯，翌日の午前７時点灯の12時間照明である。図５はスタート時のレベルを１としたコーチゾル，CD16+/NK細胞およびU５+/B細胞の相対的変化を11頭の平均値と標準誤差で示したものである。結果は省略するが，末梢血中のリンパ球数と顆粒球数には典型的な日内変動が認められ，夜間にリンパ球は上昇し顆粒球は減少する。また主要リンパ球サブセット（CD４+/T細胞，CD８+/T細胞，CD20+/B細胞，CD16+/NK）のレベルでは，図５に示すように

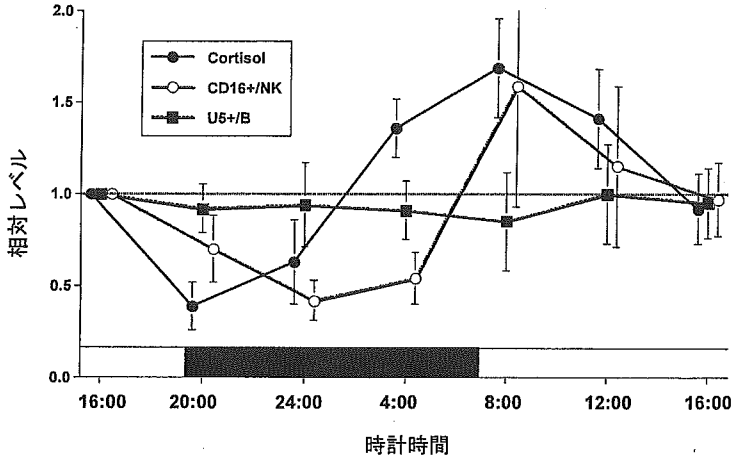

図５　ストレス関連指標の日内変動

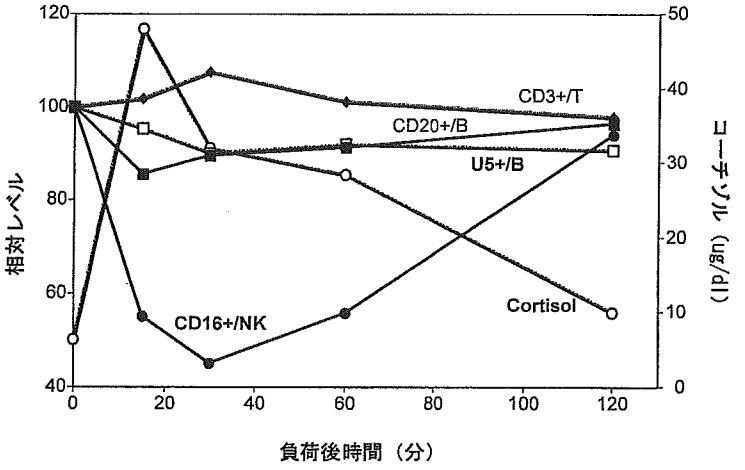

図６　コーチゾル負荷に伴う末梢リンパ球サブセットレベルの変化

CD16+/NK細胞だけが典型的な日内変動を示したが他の３種のサブセットレベルにはほとんど変化がみられなかった。図５に示すように，コーチゾルとCD16+/NK細胞レベルには典型的な日内リズムがあり，両者ともに消灯後に低下し，点灯前から翌朝にかけて増加する。この結果は，ヒトで報告されているコーチゾル及びリンパ球サブセットの日内変動とほぼ一致し，翌朝の活動開始に備えコーチゾルの増加とNK細胞の末梢血中への回帰が始まると考えられる。血中コーチゾルとCD16+/NK細胞の変化を比較すると，両者とも同一のパターンを描くが，CD16+/NK細胞の増加に先だってコーチゾルが増加していることがわかる。このことは，CD16+/NK細胞の増加は血中コーチゾルの増加により誘導される可能性を示している。すなわち，コーチゾルレベルがNK細胞レベルに影響を与えている可能性である。一方，U５+/B細胞のレベルには24時間を通じてほとんど変化がみられないことから，CD16+/NK細胞とは異なる支配を受けていることが予想された。そこで，この仮説を証明するため，チター装置を用いてコーチゾル負荷実験を行った。３頭のニホンザルにチター装置を装着し，300ug/kgのコーチゾルもしくは生理食塩水を無拘束状態で静注し，投与前，投与後15，30，60，120分に採血し，コーチゾル負荷に伴う主要リンパ球サブセットレベルの変化を調査した。図６はその一例を示したものであるが，コーチゾル静注直後からCD16+/NK細胞のみが著しく低下するが，コーチゾルレベルの低下に伴い120分後には負荷前の値に回復した。一方，U５+/B細胞レベルはCD３+/T細胞，CD20+/B細胞レベルと同様に血中コーチゾルの増加の影響をほとんど受けなかった。このことから，主要リンパ球サブセットではCD16+/NK細胞のみがコーチゾルの影響を受けるこ

とが明らかとなったが，NK細胞の変化がコーチゾルの直接的影響によるものか，より高次の神経内分泌機構がCD16+/NK細胞レベルとコーチゾルレベルの両者を支配しているかはこの結果だけでは確言し得ない。

CD16+/NK細胞レベルの変化を図5と図6で比較すると，図5の結果ではコーチゾルレベルの上昇に伴いCD16+/NK細胞レベルも増加しているが，図6の結果ではコーチゾルレベルの増加に伴いCD16+/NK細胞レベルは低下している。図1の母子分離の結果では，図6と同様に分離一日目にコーチゾルレベルが上昇するとともにCD16+/NK細胞レベルは低下している。これまでの我々の調査結果ではストレス負荷後にコーチゾルは常に増加するが，CD16+/NK細胞レベルは増加する場合と低下する場合があることが明らかになっている。前者(NK細胞レベルの上昇)は群形成やケージ交換などの比較的低レベルの持続性ストレス負荷時にみられ，後者は母子分離などの一過性の比較的高レベルのストレス反応時でみられる。これまでの結果を総合して，ストレス負荷後のNK細胞レベルの変化に関して次のような仮説を立てている。すなわち，ストレス反応として生じるコーチゾルレベルの増加の程度，換言すればストレスの程度により，CD16+/NK細胞レベルは少なくとも増加と，減少という全く異なった変化を示す。増加と減少のいずれかを決定するコーチゾルレベルの閾値が存在する可能性が高いが，閾値は個体によって異なる。この仮説を実証するため，コーチゾル濃度を変えて負荷試験を行ったが，低濃度のコーチゾル負荷ではNK細胞レベルは変化しないことから，CD16+/NK細胞レベルの増加は血中コーチゾルレベルの上昇が一定期間持続する必要があるのかもしれない。

おわりに

サル類の特性を利用してこれまでに取り組んできた神経・内分泌・免疫相関を解析するストレスモデルと，ストレスを評価する免疫学的指標について概説した。ここに示した結果はすべて複数の個体の平均値である。サル類を用いたこの種の実験では，平均値で傾向を示すよりも，個体毎のデータを比較しストレス反応の個体差を解析する方が有意義であるという気持ちをいつも持っている。個体レベルでのトータルな表現型の変化を解析する必要性である。ストレスで誘導される恒常性の撹乱は1対1の対応で判断できる単純なものではなく，いくつかの経路が複雑に関連している総合的な制御系の変化であることから，従来の還元的解析法ではなく複雑系を解析する知恵が必要となる。その意味では，個体の特性を日常的に観察している実験動物技術者の21世紀の生命科学に果たす役割は大きい。ここで紹介したストレスモデルの実験結果は，動物福祉の観点から日常的にストレス軽減に頭を悩まされている技術者の方々が動物の反応性の多様性を認識される良いきっかけにもなろう。単純系から複雑系解析に移行しつつある生命科学の分野において，個体の反応性がいくつかの制御系で統御されていることを身を持って体験されている実験動物技術者のセンスが21世紀の生命科学を支える基本であるという意を強くしている。

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Buffalo Rat Liver Cells Produce Factors that Support Preimplantation Development of Mouse Embryos Cultured In Vitro

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To examine the effects of buffalo rat liver (BRL) cells on the preimplantation development of mouse embryos in vitro, we first cultured two-cell mouse embryos alone in serum-free Dulbecco modified Eagle medium. As expected, the embryos did not develop to subsequent stages. However, when cocultured with BRL cells, the embryos developed to the blastocyst stage efficiently. Direct contact of embryos with BRL cells was not necessary for development: the medium conditioned by BRL cells contained soluble factors that supported the preimplantation development of mouse embryos. Embryos cultured with BRL-conditioned medium that was replaced at various intervals had a further increased rate of development to the blastocyst stage. This finding indicated that the activities of the factors were maintained only briefly. Seven proteins between 35 and 44 kDa that were detected in the medium were highly beneficial to the development of the embryos. Follistatin-related protein and pigment epithelium-derived factor are believed to be the factors supporting embryo development. The other five proteins also may improve the environment for the development of mouse embryos cultured in vitro.

Preimplantation development of mammalian embryos is affected by several environmental factors. In the reproductive tract, soluble factors produced by the epithelium of the fallopian tube (13, 15, 19) and uterus (3, 4, 16, 19, 22) are important for embryo development before implantation. Fertilization and embryo development to the blastocyst stage in vitro are attained in human, mouse, hamster, and rat by using chemically defined media. It is also possible to obtain a baby by transferring these embryos to a uterus (1). However, the culture system is not complete, because the rates of embryo development and time to develop to the blastocyst stage are clearly different from those in vivo. In many species it is difficult to culture an embryo in vitro. For embryo culture in these species, commercial cell culture media often are used, with modifications by each researcher. For example, serum, such as fetal calf serum, is added in the hope that unknown factors contained in it will aid embryo development. For the same reason, somatic cells (e.g., Vero, STO, and MDBK cell lines) that are easy to obtain and culture have been used as feeder cells for embryo culture (11, 12, 17). Oviduct epithelial cells, which participate in maintaining the environment for embryo development in vivo, also are used as feeder cells. Therefore, some factors produced by these cells are expected to have an effect on embryo development.

Buffalo rat liver (BRL) cells originating in rat liver are known to produce differentiation-inhibiting activity (7, 21). They are also used for embryo culture as feeder cells and have been reported to increase the rate of embryo development (14, 26). Many factors are produced by BRL cells and secreted into the culture medium (2, 8, 21). It is very important to identify these factors, because such identification can further define culture conditions that support embryo development and can lead to increased understanding of the mechanism of embryo development.

The aim of this study was to examine the effect of BRL cells on the development of preimplantation mouse embryos in vitro and to analyze the protein factors produced by BRL cells. Because supplemental serum also contains proteins, we first cultured mouse embryos in serum-free Dulbecco modified Eagle medium (DMEM) and then cultured them in the same medium with BRL cells to determine the effect of BRL cells on embryo development. Although BRL cells also affect the development of bovine (14) and monkey (26) embryos, we selected mouse embryos for this system because large numbers of embryos are required to carry it out. We subsequently analyzed the BRL cell factors at a molecular level.

Materials and Methods

Animals. Specific-pathogen-free BDF1 mice (age, 8 weeks) were purchased from Charles River Japan, Inc. (Yokohama, Japan). Microbiological monitoring for the following agents was carried out by the company: Sendai virus, mouse hepatitis virus, *Corynebacterium kutscheri*, Tyzzer's organism, *Salmonella* spp., *Pseudomonas aeruginosa*, *Pasteurella pneumotropica*, *Bordetella bronchiseptica*, *Escherichia coli 0115*, and *Mycoplasma pulmonis*. Mice had negative test results for all of the mentioned infective agents. The mice were housed in cages (35 × 29 × 18 cm) in an air-conditioned room in which the temperature (20 to 25°C) and lighting (lights on, 5:00 to 19:00) were controlled. A commercial diet (Type MF, Oriental Yeast Co., Ltd., Tokyo, Japan) and water were provided for the mice and rats ad libitum.

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For all of the experiments involving animals, the protocols were in compliance with the guidelines stated by the National Institutes of Infectious Diseases of Japan for the care, use, and biological hazard countermeasures of laboratory animals.

Preparation of culture medium conditioned by BRL cells. Frozen BRL cells were purchased from RIKEN Cell Bank (Ibaraki, Japan). Approximately 1×10^6 cells were thawed in water at 37°C for 60 sec and then resuspended in 15 ml of DMEM (GIBCO BRL Life Technologies, Grand Island, N.Y.) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin G, and 100 µg/ml streptomycin sulfate (DMEM + 10% FBS). The suspension was centrifuged at 750 ×*g* for 10 min at 4°C (RL-101, Tomy Seiko, Tokyo, Japan), and the supernatant was discarded. The pelleted BRL cells were resuspended in 15 ml of DMEM + 10% FBS, placed in a tissue culture flask (80 cm^2), and kept at 37°C in humidified 5% CO_2 in air; the medium was refreshed every 72 h. The BRL cell cultures reached confluence in the flasks within 5 to 7 days and were dispersed using trypsin–EDTA solution (Invitrogen, Carlsbad, Calif.) to make subcultures or add to wells of culture plates. When 95% of all cells had detached from the bottom of the flasks, 16 ml of DMEM + 10% FBS was added to suspend the cells; 500 µl of this suspension was added per well of 24-well plates (diameter of each well, 15 mm; no. 3526, Costar, Corning, N.Y.) and cultured for 24 h to make confluent BRL cell monolayers.

DMEM + 10% FBS was removed from the monolayers by rinsing four times with DMEM to remove serum and then adding 500 µl of DMEM. The medium was conditioned on BRL cell monolayers for 24 h. Culture wells were used for embryo coculture, or conditioned medium in the wells was used for embryo culture after filtration through a 0.22-µm filter (Millex-GP, Millipore, Bedford, Mass.). Some of the conditioned medium was passed through a centrifugal filter unit (Microcon YM-3, -10, -30, -50, or -100; Millipore) to remove substances of more than 3, 10, 30, 50, or 100 kDa and then was used for embryo culture.

Preparation of mouse embryos. Female mice were superovulated by intraperitoneal injection of 7.5 IU of equine chorionic gonadotropin (Serotropin; Teikoku Hormone, Tokyo, Japan) at 19:00 followed by 7.5 IU of human chorionic gonadotropin (Gonatropin; Teikoku Hormone) after 48 h. Fourteen hours after the second injection, the females were euthanized by cervical dislocation, and their oviducts were removed. The oocyte–cumulus complexes were isolated in a sterile culture dish containing 2 ml of TYH medium (23). Epididymal sperm were collected from male mice and capacitated for 30 min in TYH medium. A final concentration of approximately 1×10^6 sperm/ml (5-µl) was added to 50-µl drops of TYH medium containing oocytes covered with mineral oil (lot no. 211091, Mineral Oil USP heavy, Humco Texarkana, Tex.) for insemination in a sterile culture dish. The sperm and oocytes were kept together for 24 h at 37°C in humidified 5% CO_2 in air. Subsequently, some embryos were transferred to 50-µl drops of Whitten medium (25) covered with mineral oil and cultured for 96 h at 37.5°C under 5% CO_2 in air.

Examination of effects of BRL cells on preimplantation embryo development. Two-cell embryos were cultured for 96 h under one of the following conditions: 1) on a BRL cell monolayer; 2) on an insert (Millicell-CM, Millipore) placed on a BRL cell monolayer; 3) in BRL-conditioned medium; 4 through 7) in BRL-conditioned medium replaced every 3, 6, 12, or 24 h with newly conditioned medium; 8 through 12) in BRL-conditioned medium in which substances larger than 3, 10, 30, 50, or 100 kDa had been removed; or 13) in DMEM alone, without BRL cells. All embryos were cultured on 24-well plates at 37°C in humidified 5% CO_2 in air. When 24-well plates were used for embryo culture, the medium was not covered with mineral oil.

Embryos were examined daily by using an inverted microscope (Leica, Heerbrugg, Switzerland) with Hoffman Modulation Contrast optics.

Analysis of protein factors secreted by BRL cells. Ten microliters of conditioned medium was boiled for 5 min with 10 µl of Laemmli sample buffer containing 2-mercaptoethanol [10]. The sample was separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) on a 12.5% gel according to the method of Laemmli, and the gel was silver-stained according to the method of Oakley and colleagues (15).

BRL cell monolayers were grown on 6-well plates (no. 3516, Costar) in 1 ml of DMEM without methionine and cysteine (GIBCO BRL Life Technologies). After 30 min, the medium was replaced with fresh medium and the addition of 4.2 µl of Redivue Pro-mix L-[^{35}S] in vitro cell-labeling mix (100 µCi/ml, Amersham, Chicago, Ill.) and cultured for 4 h at 37°C in humidified 5% CO_2 in air. The medium then was discarded, and the plates were rinsed two times with DMEM, followed by the addition of 500 µl of DMEM. The medium was conditioned on BRL cell monolayers for 10 h. Conditioned medium containing labeled proteins was placed in 1.5-ml microtubes and centrifuged at 15,000 ×*g* for 10 min (MRX-150, Tomy Seiko). The supernatant was placed in new microtubes. One thousand blastocysts were cultured for 4 h in 100 µl of the supernatant at 37°C in humidified 5% CO_2 in air and then rinsed four times with PBS containing 0.5% bovine serum albumin. Subsequently, the embryos were lysed by boiling for 5 min in 10 µl of Laemmli sample buffer containing 2-mercaptoethanol. The sample was separated by SDS–PAGE on a 12.5% gel. The gel was dried and exposed to an image plate (Fuji Photo Film, Tokyo, Japan) for 2 weeks. The transferred image was visualized using a fluorescent image analyzer (FLA-2000R, Fuji Photo Film).

One milliliter of conditioned medium was concentrated to 10 µl (100 times) in a centrifugal filter unit (Centriplus YM-10, Millipore). The medium then was boiled for 5 min with 10 µl of Laemmli sample buffer containing 2-mercaptoethanol. The sample was separated by SDS–PAGE on a 12.5% gel. The gel was stained with a Coomassie brilliant blue R-250 staining kit (Bio-Rad Laboratories, Hercules, Calif.). The bands detected by Coomassie brilliant blue staining that were in the same position as the bands detected by the fluorescent image analyzer were cut out and treated with trypsin. The tryptic digests were analyzed directly by using liquid chromatography–tandem mass spectrometry. The analysis was performed by using a C_{18} column (Magic C18, 0.1 × 50 mm; Michrom BioResource, Auburn, Ala.) coupled to a tandem mass spectrometer (Q-Tof2, Micromass, Manchester, United Kingdom) equipped with a nanoelectrospray ionization source. Positive ion tandem mass spectra were then measured. The results were searched on the Mascot database.

Statistical analysis. Significant differences in the number of embryos in each group that developed to the blastocyst stage were assessed using a chi-square test and statistical significant difference was considered to be $P < 0.05$ (STATISTICA, StatSoft Japan Inc., Tokyo, Japan).

Table 1. Effect of coculture with buffalo rat liver cells on mouse embryo development

Conditions of embryo culture	No. of embryos cultured	No. (%) of embryos that developed to			
		2- to 3-cell	4-cell	morula	blastocyst
Coculture on feeder cells	113	0 (0)	0 (0)	6 (5)	107 (95)[a]
Coculture on insert	92	1 (1)	0 (0)	11 (12)	80 (87)[a]
DMEM alone	227	221 (97)	6 (3)	0 (0)	0 (0)

[a]No significant differences were found between values indicated with the same superscript letter ($P > 0.05$).

Table 2. Effect of interval at which conditioned medium was replaced on mouse embryo development

Interval at which conditioned medium was replaced (h)	No. of embryos cultured	No. (%) of embryos that developed to			
		2- to 3-cell	4-cell	morula	blastocyst
3	86	0 (0)	2 (2)	17 (20)	67 (77)[a]
6	142	4 (3)	1 (1)	44 (31)	93 (65)[a]
12	85	14 (14)	4 (4)	19 (22)	48 (56)[ab]
24	67	3 (4)	2 (3)	36 (54)	26 (39)[bc]
No replacement	198	50 (25)	18 (9)	70 (35)	60 (30)[c]

[a,b,c]No significant differences were found between values indicated with the same superscript letter ($P > 0.05$).

Table 3. Effect of removal of substances of differing molecular mass on mouse embryo development

Molecular mass of substances removed from conditioned medium (kDa)	No. of embryos cultured	No. (%) of embryos that developed to			
		2- to 3-cell	4-cell	morula	blastocyst
No removal	198	50 (25)	18 (9)	70 (35)	60 (30)[a]
> 100	37	0 (0)	0 (0)	25 (68)	12 (32)[a]
> 50	36	4 (11)	0 (0)	19 (53)	13 (36)[a]
> 30	37	1 (3)	0 (0)	32 (86)	4 (11)[b]
> 10	36	12 (33)	0 (0)	24 (67)	0 (0)
> 3	36	21 (58)	3 (8)	12 (33)	0 (0)

[a,b]Significant differences were detected between values indicated with the different superscript letter ($P < 0.05$).

Results

Two-cell mouse embryos cultured in serum-free DMEM did not develop at all. When cultured with the same medium but with BRL cells, all embryos developed to the morula stage, and 95% developed to the blastocyst stage. When cultured on an insert that prevented direct contact with the BRL cells, 99% of the embryos developed to the morula stage, and 87% developed to the blastocyst stage (Table 1).

When cultured with conditioned medium that was replaced every 3, 6, 12, or 24 h with newly conditioned medium, 97%, 96%, 78%, and 93% of the embryos developed to the morula stage, and 77%, 65%, 56%, and 39% of embryos developed to the blastocyst stage, respectively. When cultured with conditioned medium that was not replaced, 35% of the embryos developed to the morula stage, and 30% developed to the blastocyst stage (Table 2).

When cultured with conditioned medium from which substances greater than 100, 50, 30, 10, or 3 kDa had been removed by passage through a centrifugal filter unit, 100%, 89%, 97%, 67%, and 33% of embryos developed to the morula stage, and 32%, 36%, 11%, 0%, and 0% of embryos developed to the blastocyst stage, respectively (Table 3).

As a result of SDS–PAGE and subsequent silver staining of the medium conditioned by BRL cells, many bands were observed (Fig. 1). Bands representing proteins contained in ^{35}S-methionine-labeled conditioned medium that combined with or were incorporated into a mouse embryo were observed between 35 and 44 kDa (Fig. 2). The proteins, containing the peptide fragments detected by liquid chromatography–tandem mass spectrometry analysis of their amino acid sequences were as follows: actin gamma, acyl coenzyme A hydrolase, capping protein (actin filament), follistatin-related protein, pigment epithelium-derived factor, phosphoglycerate kinase (EC 2.7.2.3), and transcobalamin II precursor (Table 4).

Discussion

In this study we showed that BRL cells secrete factors into culture medium that support development of preimplantation mouse embryos in vitro. We believe that several factors produced by BRL cells provide this synergistic effect. A factor inhibiting embryo development is produced by another cell line (9). Thus, such factors may be among the factors produced by BRL cells.

Our findings clarify the contribution of proteins produced by BRL cells to embryo development. Preimplantation embryos cultured with BRL-conditioned medium that was replaced at shorter intervals developed into blastocysts at a higher rate than did those cultured with conditioned medium that was not replaced (Tables 1 and 2). This shows that the proteins do not have stable molecular structure or that their effects on embryo development were easily lost during the culture period. It is also possible that the effectiveness of each factor differs at different developmental stages of the embryos or that the required factors are insufficient once metabolized with the embryos. Although there were no significant differences in the development of preimplantation embryos into blastocysts cultured with BRL-conditioned medium, medium from which > 50-kDa materials were removed, and medium from which > 100-kDa materials were removed, the development of the embryos cultured with medium from which materials > 30 kDa were removed was significantly suppressed. The development of preimplantation embryos cultured with medium from which > 3- or

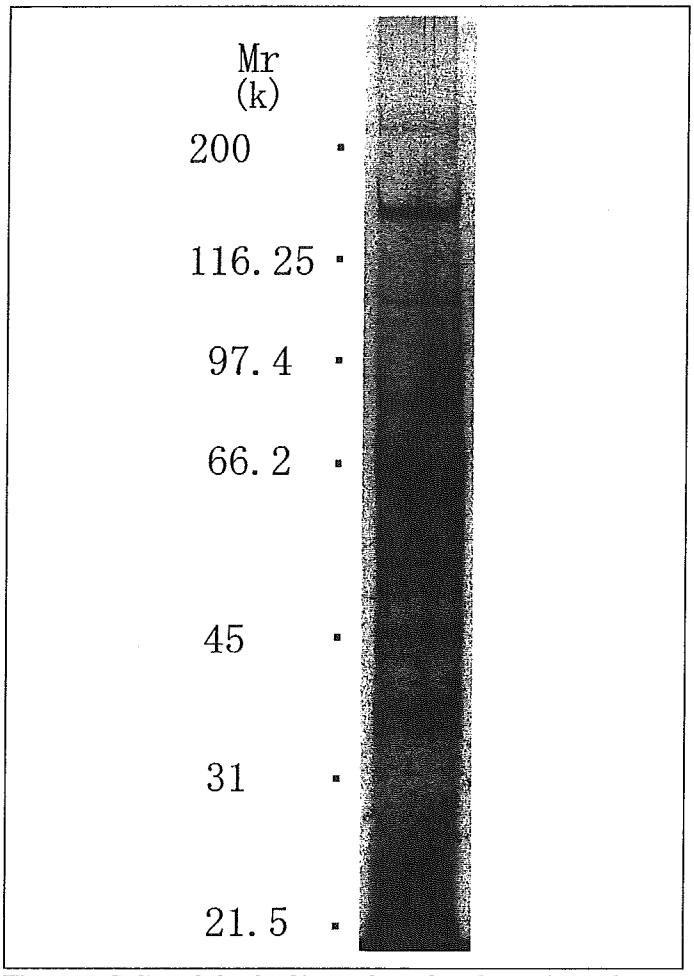

Figure 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of proteins contained in the medium conditioned by BRL cells. Proteins were separated on a 12.5% gel and then silver-stained.

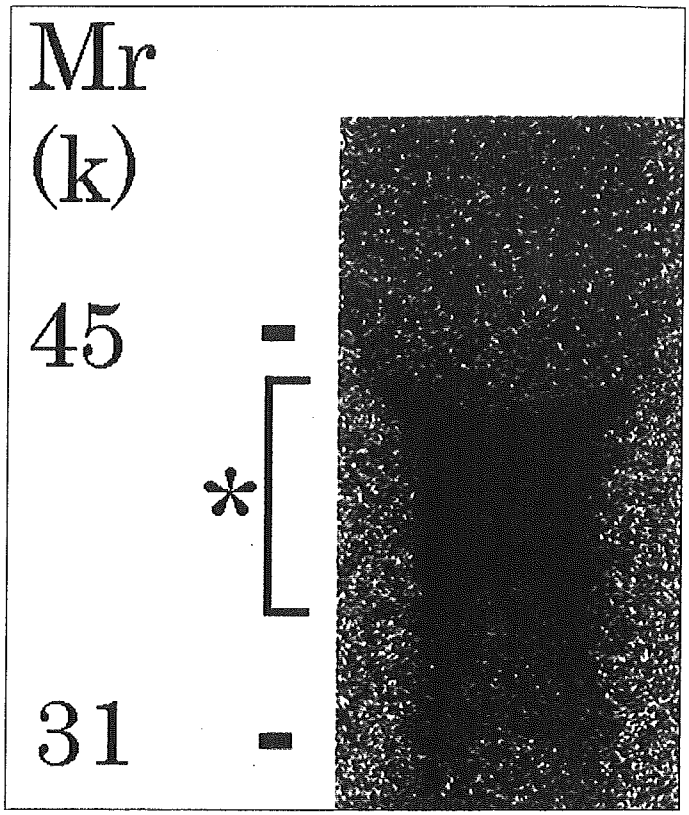

Figure 2. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of ^{35}S-labeled proteins that combined with or were incorporated into a mouse embryo and those secreted by BRL cells. The proteins were separated on a 12.5% gel and then detected by a fluorescent image analyzer. Bands were observed in the region between 35 and 44 kDa. The asterisk indicates the position equivalent to that observed by means of liquid chromatography–tandem mass spectrometry in a subsequent experiment.

> 10-kDa materials were removed was completely suppressed (Table 3). The rates of development of preimplantation embryos into blastocysts in the experiments of Tables 1, 2, and 3 were rather different because of the details of experiments were different, e.g., frequency of medium change. However, these results indicate that the promoting protein(s) for embryo development were between 10 and 50 kDa in size.

We confirmed that the proteins strongly combining with or incorporated into mouse embryos are in the range of 35 to 44 kDa. Seven proteins were identified in this range: actin gamma, acyl coenzyme A hydrolase, capping protein (actin filament), follistatin-related protein, pigment epithelium-derived factor, phosphoglycerate kinase (EC 2.7.2.3), and transcobalamin II precursor (Table 4). Follistatin, which combines strongly with activin, has been reported to suppress the promotion of bovine embryo development by neutralizing activin (20). Although the effect of follistatin-related protein on the embryos in our study is unclear, the activity of this protein has been reported to similar to that of follistatin (24). On the other hand, activin induces apoptosis in some kinds of cells (6). Therefore, in the culture environment that did not sustain mouse embryos, follistatin-related protein may have acted indirectly to suppress the induction of apoptosis by neutralizing activin. The effect of pigment epithelium-derived factor on embryo development is still unclear, although its influence on embryos has been reported previously (18). The remaining five factors also may have influenced embryo development, although improvement of embryo development in vitro by their addition to culture medium has not been reported previously.

In conclusion, in this study we analyzed the factors produced by BRL cells and showed the possibility of their participation in embryo development. This analysis was made possible by a culture system that uses serum-free culture medium, a technique that should also be effective for the molecular analysis of other proteins in medium. To explore the action and mechanism of such proteins, it is necessary to identify the proteins effective in embryo development and to examine embryo development in culture medium to which one or more proteins have been added. This research could become the foundation for resolving many mysteries about preimplantation embryo culture.

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