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MHC Class I Alleles Influence Set-Point Viral Load and Survival Time in Simian Immunodeficiency Virus-Infected Rhesus Monkeys[1]

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In HIV-infected humans and SIV-infected rhesus macaques, host genes influence viral containment and hence the duration of the disease-free latency period. Our knowledge of the rhesus monkey immunogenetics, however, is limited. In this study, we describe partial cDNA sequences of five newly discovered rhesus macaque (*Mamu*) class I alleles and PCR-based typing techniques for the novel and previously published *Mhc* class I alleles. Using 15 primer pairs for PCR-based typing and DNA sequence analysis, we identified at least 21 *Mhc* class I alleles in a cohort of 91 SIV-infected macaques. The results confirm the presence of multiple class I genes in rhesus macaques. Of these alleles, *Mamu-A*01* was significantly associated with lower set-point viral load and prolonged survival time. *Mamu-A*1303* was associated with longer survival and a "novel" *Mhc* class I allele with lower set-point viral load. The alleles are frequent in rhesus macaques of Indian origin (12–22%). In addition, survival probability of individual SIV-infected rhesus monkeys increased with their number of alleles considered to be associated with longer survival. The results contribute to improve the interpretation and quality of preclinical studies in rhesus monkeys. *The Journal of Immunology*, 2002, 169: 3438–3446.

Simian immunodeficiency virus-infected rhesus macaques represent, to date, the best animal model for preclinical studies into preventive vaccines and novel therapies for AIDS. After infection with SIV, rhesus macaques develop a disease similar to HIV-1-induced AIDS. Like in HIV-infected humans, the monkeys display a considerable interindividual variability with respect to control of viral replication. This in turn confounds the interpretation of experiments using outbred monkeys. The small number of animals per experiment also does not always support a rigorous statistical analysis (1). Particularly, when only a reduction of the viral burden resulting from immunization is taken as end point, convincing quantification of the results may be hard to achieve. Subtle effects of an improved formulation, although potentially beneficial, may not be assessed at all. The design and a more reliable interpretation of the experiments could be improved significantly by assigning only immunobiologically similar monkeys to the treated and the untreated control group.

Potentially, such genetically similar animals could be produced by assisted reproductive technology (2). However, at the moment there are no capacities for a fast large-scale production of such animals in most primate centers. Currently, it would be more feasible to select animals before the onset of an experiment according to predictive genetic properties. Especially the knowledge of genetic markers would allow a routine screening of large numbers of animals and would also facilitate conventional as well as assisted breeding strategies.

In addition, a preclinical animal disease model, relevant for humans, should mimic to some extent the genetic variety in humans. Ideally, this genetic diversity should not be a black box, but should be investigated and manageable. A prerequisite is obviously a knowledge of either the genes influencing the disease course or linked marker sequences. In this respect, the polymorphic *Mhc* genes are particular valuable, since they play a crucial role during the initiation and regulation of an immune response. Numerous reports on the association of *Mhc* genes with disease progression in HIV-1-infected humans have been published. Some associations have been repeatedly detected, e.g., HLA-B*14, -B*27, and -B*57 in slow progressors and HLA-B*22, HLA-B*35, and HLA-B*44 in humans with shorter survival time (3–10). Some of these alleles might also influence cytotoxic T cell responses to vaccines (11). Extended MHC haplotypes which include TAP genes and chemokine coreceptor genes are also known to influence disease progression in HIV-1-infected humans (4, 6, 12–14) Furthermore, heterozygosity or the presence of MHC molecules able to present many different HIV-1-derived peptides positively influence the disease course in HIV-1-infected humans (7, 15, 16).

Mhc genes and haplotypes associated with disease progression have also been identified in SIV-infected rhesus monkeys (17–19). Since the virus strains used for most experiments in SIV research are genetically less divers than the different HIV-1 subtypes, one would expect more consistent associations between *Mhc* alleles and disease progression than in HIV-1-infected humans.

A major obstacle in determining the role of *Mhc* class I polymorphisms for disease progression in SIV-infected rhesus monkeys is the lack of molecular typing techniques for class I molecules. This reflects a lack of detailed knowledge about the MHC class I region in rhesus macaques. So far, molecular typing techniques have been described only for a few MHC class I molecules (20–22). In addition, there are remarkable differences between the organization of the human and the macaque *Mhc* class I region. In contrast to humans, rhesus macaques may possess one to three *A*

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and two to four *B* locus genes (Refs. 23 and 24; unpublished results). One of the classical *B* genes seems to be highly conserved and was termed *Mamu-I* (25). The presence of multiple class I genes may be common to all members of the subtribe *Papionina*, but their biological significance is unknown (23, 26, 27).

The aim of this study was to identify *Mhc* class I alleles in rhesus macaques, to develop *Mhc* class I typing techniques for novel and published alleles, and to identify *Mhc* class I alleles associated with disease progression in SIV-infected macaques. The results can be used to improve the design and the interpretation of experiments performed in rhesus monkeys.

Materials and Methods

Animals and viruses

Samples were collected from 91 naive SIV-infected animals housed at the German Primate Center according to the German animal protection act. Data from most of the animals had been previously published (19, 28–32). Thirty-seven animals were infected with SIVmac 251 grown on monkey PBMCs (33, 34). Ten monkeys were infected with SIVmac 251/32H grown on the human T cell line C8166 (35). Thirteen rhesus monkeys were infected with the ex vivo isolate SIVmac251/32H/spl (36).

Twenty-nine monkeys were infected with SIVmac239 grown on rhesus monkey PBMCs (37–39). Two animals were infected with supernatant from SIVmac239-transfected COS cells (40). Forty monkeys were obtained from the German Primate Center breeding colony, originally derived from the Caribbean Primate Research Center colony, Cayo Santiago (Puerto Rico), 15 directly from the Carribbean Primate Research Center, and 36 from Laboratory Animal Breeders and Services of Virginia.

Nucleic acid extraction and synthesis of Mhc *class I* A *and* B *locus-specific PCR products*

DNA was extracted by standard salting-out techniques using either B cells, lymphocytes, or frozen tissue samples. RNA was extracted from 2×10^6–2×10^7 B cells or PBMC using the RNeasy Midi-Kit (Qiagen, Hilden, Germany). For reverse transcription, 4 μg of total RNA, 2 μg of random hexamers (Invitrogen, Carlsbad, CA), and a suitable volume of distilled H_2O were incubated at 70°C for 10 min. Forty units of RNasin (Promega, Mannheim, Germany), 1 mM dNTP, 10 mM DTT, 1× first-strand buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM $MgCl_2$), and 600 U of Superscript II RNase H^- reverse transcriptase (Invitrogen) were added to give a final volume of 40 μl. cDNA was synthesized at room temperature for 10 min, at 42°C for 30 min, and at 70°C for 10 min. cDNA was purified and concentrated by phenol-chloroform extraction and a subsequent ethanol precipitation. The 50-μl PCR mixture contained 1/10 volume of the purified cDNA, 20 pmol of MHC class I A-(*A-DGGE-for* and *rev*) or B-specific denaturing gradient gel electrophoresis (DGGE)[3] (*B-DGGE-for* and *rev*) primers (Table I), 200 μM of each deoxyribonucleotide triphosphate (Peqlab, Erlangen, Germany), 1× PCR buffer, 0.5 M GC-rich resolution solution, and 1 U of GC-Rich-PCR System Enzyme Mix (Roche Diagnostics, Mannheim, Germany). The reactions were denatured initially at 95°C for 3 min followed by 30 cycles of 95°C for 30 s, 60°C (A-DGGE primer) or 62°C (B-DGGE primer) for 30 s, 72°C for 40 s, and a single final extension step at 72°C for 3 min. Primers used were synthesized and HPLC purified by Sigma-ARK (Darmstadt, Germany).

DGGE

Thirty microliters of the PCR products were mixed with 15 μl of loading buffer and separated by electrophoresis in a 10% (37.5:1 acrylamide:bisacrylamide; Bio-Rad, Munich, Germany) polyacrylamide gel with an increasing gradient of 50–65% denaturant (100%: 7 M urea, 40% formamide; Bio-Rad D-Gene apparatus, Bio-Rad). DGGE analysis was performed at 130 V and 56°C for 9 h in 1× Tris-acetate-EDTA buffer. After the run, bands were visualized with SYBR-Green (Biozym, Hess. Oldendorf, Germany) and excised with sterile scalpels. DNA was eluted overnight in 50 μl of distilled water at room temperature. Five microliters of the eluate was mixed with 15 pmol of sequencing primers (MWG Biotec, Munich, Germany; see Table I) in a 50-μl reaction volume containing 200 μM dNTP (Peqlab), PCR buffer and 1 U GC-Rich-PCR System Enzyme Mix (Roche Diagnostics). The DNA was denatured initially for 3 min at 95°C. Amplification was performed for 30 cycles at 95°C for 30 s, at 60°C (*A-Seq*) or 62°C (*B-Seq*) for 30 s, and at 72°C for 30 s, followed by a final elongation step at 72°C for 3 min. PCR products were purified on spin columns (Millipore, Eschborn, Germany) and sequenced by SeqLab (Goettingen, Germany) using standard M13 forward and reverse primers. The DNA sequences described in this article have been submitted to GenBank and have been assigned the following accession numbers: NA4, AF518412, NA7, AF519897, NB2, AF519898, NB4, AF519899, NB5, and AF519900. For registration, the DNA sequences have been sent to the Primate MHC register, but have not yet been registered. The designations are therefore arbitrary.

Typing techniques

Thirteen rhesus monkey allele-specific primer pairs (18–22 mer) were designed based on the published and the novel DNA sequences. The specific primers for amplification of the *Mhc* class I *A* and *B* locus alleles are listed in Table I. Allele-specific PCR products ranged in size from 408 to ~1300 bp. MHC *DRB* gene-specific primers (*MDRB5′* and *3′MDR*) served as an internal control (20). Primers were synthesized and purified by a high-purity salt-free method (HPSF) from MWG Biotec. A reaction mixture of 25 μl contained 70–110 ng DNA, PCR buffer, Q-solution, 200 μM dNTP, 15–20 pmol allele-specific 5′ and 3′ primer, 2–10 pmol of the DRB primers, and 1.25 U of *Taq* polymerase (Qiagen). The samples were denatured initially at 95°C for 3 min followed by 30–37 cycles at 95°C for 30 s, 58–63°C for 40 s, 72°C for 40 s, and a final elongation step at 72°C for 3 min (Table II). Allele-specific products were identified by electrophoresis of amplicons in 1% agarose gels containing ethidium bromide. For the preparation of the sequencing samples, the PCR was performed as described above, except for the omission of the internal control primers. Before sequencing, the PCR samples were directly purified on spin columns (Microcon-PCR; Millipore) or extracted from an agarose gel electrophoresis using centrifugal filters (Ultrafree-DA; Millipore). DNA sequencing was performed by SeqLab using standard M13 forward and M13 reverse primers.

Quantification of viral RNA

A highly sensitive, single-state QC RT-PCR technique has been developed and validated for the quantification of $SIV_{mac/sm}$ RNA in plasma (41). The sensitivity and dynamic range of this assay are such that it is capable of detecting 40 copies to 4×10^8 copies of SIV RNA per ml of plasma. In brief, RNA is extracted from 200 μl of serum or EDTA plasma using guanidine isothiocyanate-mediated lysis, followed by propanol-2 precipitation of the RNA. A known amount of synthetic internal standard RNA is added before the RNA purification and is copurified to monitor the efficiency of the purification. The RNA is reverse transcribed and amplified in a single reaction protocol using rTth DNA polymerase (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and biotinylated primers. The internal standard RNA is coamplified to monitor the amplification efficiency. The amplified fragments are detected by a capture probe that is covalently bound to Nucleolink microwells (Nunc, Roskilde, Denmark). The amplification products are detected by a streptavidin-HRP-mediated colorimetric reaction. The amplified internal standard is hybridized to a different capture probe in separate microwells. The number of RNA copies in the sample is calculated from the OD of the sample wells compared with that of the corresponding internal standard well.

Statistical analysis

Contingency tables were tested for a statistically significant dependence between rows and columns using either Fisher's exact test or a χ^2 test, depending upon cell counts. Means of viral load associated with different allelotypes were compared using a nonparametric Kruskal-Wallis test with χ^2 approximation. Kaplan-Meier survival analysis of animals with different allelotypes was performed using a log rank statistic for comparative purposes and log linear model fitting for parameter estimation. All statistical analyses were performed using the SAS software package (SAS version 6; SAS, Cary, NC).

Results

Mhc *class I allele frequencies and potential linkages*

Initial typing studies using DGGE and DNA sequence determination of class I-derived RT-PCR products from 14 rhesus macaques led to the identification of 5 novel class I alleles (Fig. 1). Subsequently, 15 primer pairs were developed to type the novel and previously published rhesus macaque class I alleles. The monkeys were also screened for *Mamu-A*01* and *Mamu-A*02* (20, 22). The use of primers specific for *Mamu-B*03* or *Mamu-B*04* did not reveal any positive animal in this cohort (data not shown). Testing

[3] Abbreviations used in this paper: DGGE, denaturing gradient gel electrophoresis; wpi, week postinfection.

Table I. *Primer sequences*

Primer	Primer Sequence (5′–3′)	Size of PCR Product (bp)[a]
A-DGGE-for	ATG GCG CCG CGA ACC CTC CTC CTG G	592
A-DGGE-rev	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC CTG GTG GGT CAC ATG TGT CTT G	
B-DGGE-for	ATG GCG CCC CGA ACC CTC CTC CTG C	523
B-DGGE-rev	CGC CCG CCG CGC CCC GCG CCC GGC CCG CCG CCC CCG CCC TAT CTG CGG AGC CAC TCC	
A-Seq-for	TGT AAA ACG ACG GCC AGT ATG GCG CCG CGA ACC CTC	
A-Seq-rev	CAG GAA ACA GCT ATG ACC CGC TGC AGC GTC TCC TTC	
B-Seq-for	TGT AAA ACG ACG GCC AGT AGA CCT GGG CCG GCT CGC	
B-Seq-rev	CAG GAA ACA GCT ATG ACC TAT CTG CGG AGC CAC TCC	
A01 for[b]	GAC AGC GAC GCC GCG AGC CAA	685
A01 rev[b]	GCT GCA GCG TCT CCT TCC CC	
A02 for[c]	TGT AAA ACG ACG GCC AGT GTG GGT GGA GCA GGA GGG TCC	~1300/420
A02 rev[c]	CAG GAA ACA GCT ATG ACC CAG CAC CTC AGG GTG GCC TCT	
A04 for	TGT AAA ACG ACG GCC AGT CCA TGA GCT ATT TCT ACA CCT A	668/427
A04 rev	CAG GAA ACA GCT ATG ACC GGT AGG TTC TGT GCT GCT C	
A08 for	TGT AAA ACG ACG GCC AGT CCT TGA GGT ATT TCT ACA CCG	611/370
A08 rev	CAG GAA ACA GCT ATG ACC GCA GCC ATG TCC GCT GCC	
A11 for	TGT AAA ACG ACG GCC AGT GGG AGC CCC GCT TCT TCA	579/336
A11 rev	CAG GAA ACA GCT ATG ACC TTC TGA GCC GCC ATG TCC C	
A13 for	TGT AAA ACG ACG GCC AGT ATG AGG TAT TTC TAC ACC TCC A	669/428
A13 rev	CAG GAA ACA GCT ATG ACC CCA GGT AGG CTC TCA TCC T	
NA4 for	TGT AAA ACG ACG GCC AGT TCC ATG AGG TAT TTC TAC ACC A	672/431
NA4 rev	CAG GAA ACA GCT ATG ACC CCA GGT AGG CTC TCA TCC T	
NA7 for	TGT AAA ACG ACG GCC AGT ATG AGG TAT TTC TAC ACC TCC A	606/365
NA7 rev	CAG GAA ACA GCT ATG ACC CGC CAC ATC CGC CGC GAA	
B01 for	TGT AAA ACG ACG GCC AGT ACC GGG AGA CAC GGA AGG	530/286
B01 rev	CAG GAA ACA GCT ATG ACC AGC CAC TCC ACG CAC CGG	
B07 for	TGT AAA ACG ACG GCC AGT GGT ATT TCA GCA CCG CCG TA	638/395
B07 rev	CAG GAA ACA GCT ATG ACC CAC TTG CGC TGG GTG AAC C	
B12 for	TGT AAA ACG ACG GCC AGT GCC GGG AAC CCT GGT ATC	624/381
B12 rev	CAG GAA ACA GCT ATG ACC GAA CCG CTC CGC ATA ACG GT	
B17 for	TGT AAA ACG ACG GCC AGT GCG ACA CGG AGA GCC AAG GA	482/239
B17 rev	CAG GAA ACA GCT ATG ACC CCG CTC CGC ATA ACG GTT CC	
NB2 for	TGT AAA ACG ACG GCC AGT ACT CCA TGA GGT ATT TCA CC	666/423
NB2 rev	CAG GAA ACA GCT ATG ACC CTT CTG CTC CGC CAC ACG	
NB4 for	TGT AAA ACG ACG GCC AGT GCC GGG AAC CCT GGT ATG	624/381
NB4 rev	CAG GAA ACA GCT ATG ACC GAA CCG CTC CGC ATA ACG GT	
NB5 for	TGT AAA ACG ACG GCC AGT CCC CGC TTC ATC TCC GTG	541/298
NB5 rev	CAG GAA ACA GCT ATG ACC TCG TAG GCG TCC TGC CGG	
MDRB5[d]	GCC TCG AGT GTC CCC CCA GCA CGT TTC	260
3′MDR[d]	GCC GCA GCT TTC ACC TCG CCG CTG	

[a] Size of PCR fragments with/without intron.
[b] Ref. 20.
[c] Ref. 22.
[d] *MHC DRB* gene-specific primer (17).

of the specificity of the primers by PCR was mainly performed in another monkey cohort (To Mühl, manuscript in preparation).

All PCR products produced by the NB2, NB4, NB5, A13, A11, and B17 primers were sequenced. The DNA sequence determination revealed one novel *Mamu-B*17*, one novel *Mamu- A*11*, two novel *Mamu -A*13* allelic variants, and one allele similar to *NB5*. Thus, with the primers described here, at least 21 *Mhc* class I alleles were distinguished. The PCR products of the most frequent alleles (e.g., *NA7*, *NA4*, *Mamu-A*02*, *-A*04*, and *-B*12*) were not sequenced exhaustively because the PCR products were detected in rapid- or slow-progressing animals in comparable frequencies. Hence, these primer pairs were clearly not suitable for detection of *Mamu* class I alleles associated with disease progression in SIV-infected monkeys.

Three to nine class I alleles were detected per animal. Table III shows the allele frequencies in the cohort of 91 unvaccinated monkeys with respect to their origin. As expected, the allele frequencies differed considerably. Some alleles were present in 50–80% of all animals, whereas others were present in only a few animals. Especially, *NA7* may be an allele of a highly conserved gene present in nearly every rhesus monkey of Indian origin. This allele and highly similar alleles were also detected in macaques of Chinese origin (data not shown). Recently, a highly conserved *B* gene termed *Mamu-I* has been described (25). *NA7* may be an equivalent of a highly conserved *A* gene.

Table II. *PCR conditions for rhesus monkey* Mhc *class I typing*

Mhc-Specific Primer	*Mhc* Primer Concentration (pmol/sample)	*DRB* Primer Concentration (pmol/sample)	Annealing Temperature (°C)	No. of Cycles
A01	20	4	66	35
A02	20	4	66	30
A04	18	2	61	35
A08, A11	20	4	64	35
A13	18	2	60	35
NA4	20	2.5	61	37
NA7	20	2.5	64	35
B01	20	10	62	35
B07	18	4	60	35
B12	21	3	63	35
B17	21	2	64	35
NB2	18	2	62	35
NB4	20	3	61	35
NB5	20	5	62	35

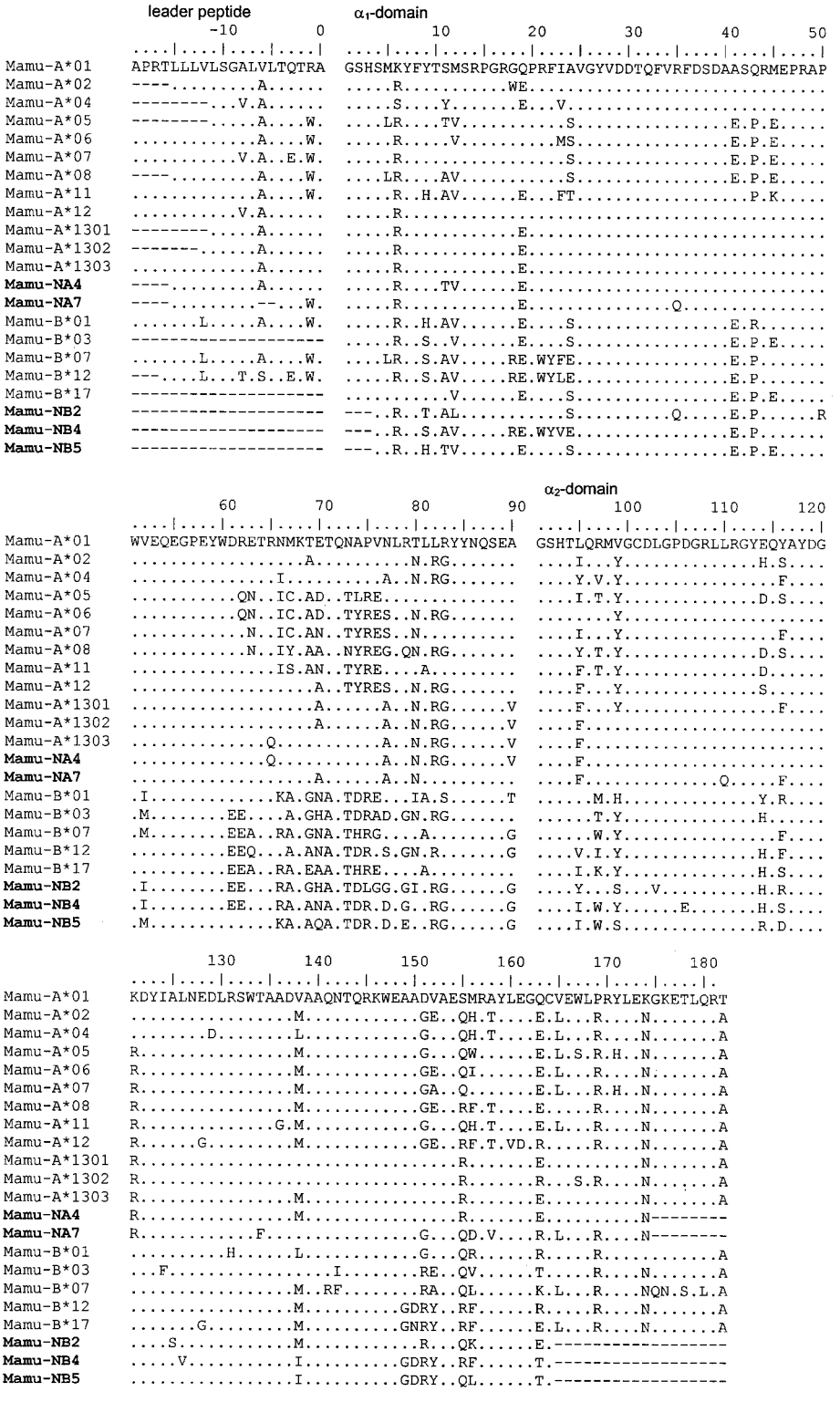

FIGURE 1. Alignment of the deduced amino acid sequences of selected rhesus monkey *Mhc* class I alleles. Amino acid sequences were aligned to the Mamu-A*01 sequence and span the leader peptide and the α_{1-3} domains (exons 2–4). Identity with Mamu-A*01 is indicated with periods, while differences are given by the conventional amino acid one-letter code. Lack of sequence information is indicated by dashes. Novel alleles are indicated in bold.

A few linked alleles were identified. *Mamu-A*08* was associated with some *Mamu-A*13* variants. It was detected in 19 of the 20 animals carrying *NA4*, which is highly similar to *Mamu-A*1303*. In addition, all 20 carriers of *Mamu-A*1303* were also positive for *Mamu-A*08* (Fisher's exact test, $p < 0.0001$). The identity of *Mamu-A*08* was validated by DNA sequence analysis of PCR products from eight animals carrying *Mamu-A*1303*, *NA4*, or *Mamu-A*08* alone. There also seems to be an association between *Mamu-A*01* and *NB5*. All but 1 of the 13 *Mamu-A*01* carriers had *NB5* (Fisher's exact test, $p < 0.0001$).

Association of MHC class I alleles with viral load and survival time

Two parameters were used to identify rhesus macaque *Mhc* class I alleles associated with disease progression: set-point plasma viral RNA load and survival time after infection. As viral set-point, either week 20 after infection was used or the time of death when the monkeys died earlier. A total of 51 animals that died of AIDS had viral load data available. Among these, a highly significant negative correlation was observed between survival time and

Table III. *Frequencies of the* Mhc Mamu *class I alleles*[a]

	Allele Frequencies					
	All animals (n = 91)		DPZ and CPRC animals (n = 55)		LABS animals (n = 36)	
Alleles	%	n	%	n	%	n
*A*01*	14.3	13	16.4	9	11.1	4
*A*02*	16.0	15	5.5	3	33.3	12
*A*04*	70.3	64	80.0	44	55.6	20
*A*08*	48.4	44	51.0	28	44.4	16
*A*11*	2.2	2	0	0	5.6	2
*A*1302*	13.2	12	3.6	2	27.8	10
*A*1303*	22.0	20	23.6	13	19.4	7
NA4	22.0	20	29.1	16	11.1	4
NA7	81.3	74	83.6	46	77.8	28
*B*01*	22.0	20	12.7	7	36.1	13
*B*07*	28.6	26	18.2	10	44.4	16
*B*12*	56.0	51	67.3	37	38.9	14
*B*17*	5.5	5	3.6	2	8.3	3
NB2	19.8	18	21.8	12	16.7	6
NB4	7.7	7	9.0	5	5.6	2
NB5	19.8	18	25.5	14	11.1	4

[a] CPRC, Carribean Primate Research Center; LABS, Laboratory Animal Breeders and Services; DPZ, German Primate Center.

plasma viral RNA load (Spearman rank correlation coefficient, $r = -0.508$, $p = 0.004$; Fig. 2).

When tested by a nonparametric Kruskal-Wallis test, only three alleles showed a significant influence ($p < 0.05$) on viral load, namely, *Mamu-A*01* ($\chi^2 = 7.3487$; $p = 0.0067$), *NB2* ($\chi^2 = 4.0809$; $p = 0.0434$), and *NB5* ($\chi^2 = 6.2770$; $p = 0.0122$). Carriers of one of the three alleles have a lower mean rank and, therefore, a lower viral load. However, as noted above, *Mamu-A*01* and *NB5* are strongly associated. Only *NB2* was not associated with either of the other two alleles.

When all 91 animals were assessed in a Kaplan-Meier analysis, four alleles exhibited a statistically significant influence on the survival time, namely, *Mamu-A*01*, *Mamu-A*08*, *Mamu-A*1303*, and *NB5*. These alleles had negative log ranks associated with them, indicating that they delayed the onset of symptoms. This is also reflected by the estimates of the mean survival time (Table IV and Fig. 3).

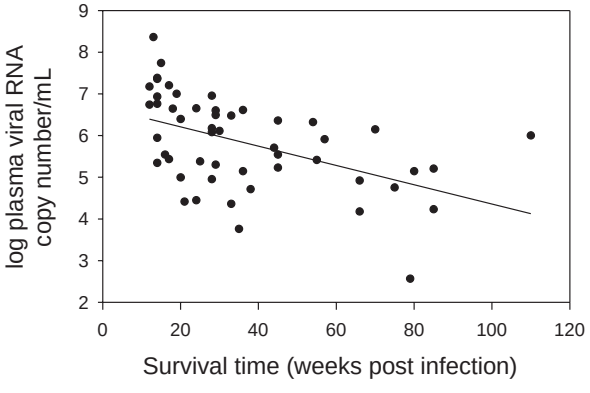

FIGURE 2. Scatter plot showing the correlation between log plasma SIV RNA load at postacute viral set point and survival time. Virus load data were available for 51 animals euthanized with clinical AIDS. Plasma samples were taken 20 wk post infection or at the time of death if the monkeys died earlier. Statistical analysis showed a significantly negative correlation between survival time and virus load (Spearman rank correlation coefficient, $r = -0.508$, $p = 0.0004$).

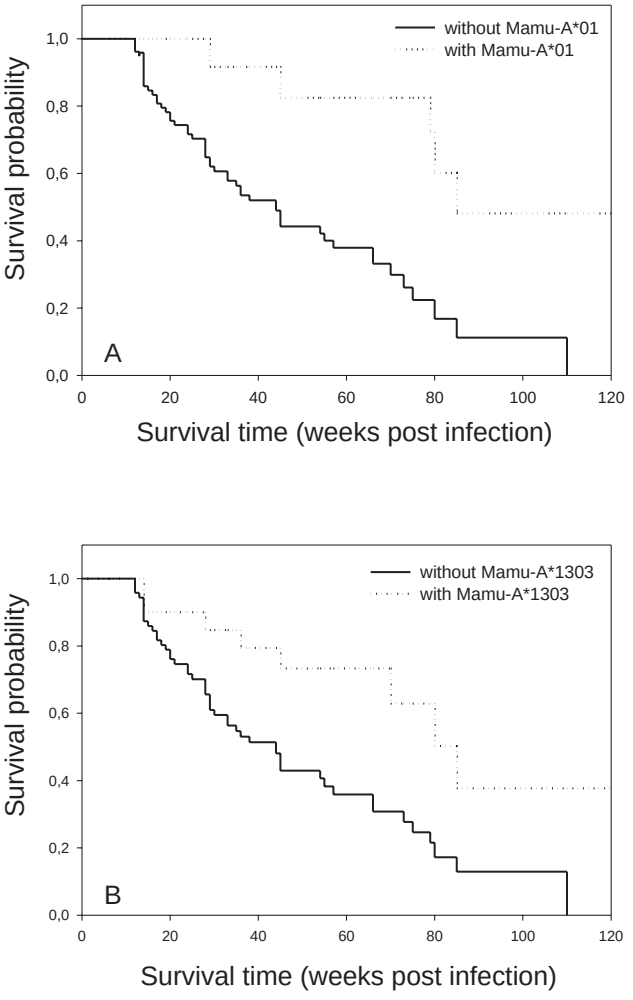

FIGURE 3. Kaplan-Meier plot of survival probability of SIV-infected rhesus monkeys according to their MHC alleles. Statistical analysis shows that the alleles *Mamu-A*01* ($p = 0.0015$) and *Mamu-A*1303* ($p = 0.0046$) are the only two independent alleles associated with longer survival. *A*, Survival probability for 13 monkeys with *Mhc* class I allele *Mamu-A*01* (dotted line) and 78 monkeys without *Mamu-A*01* (solid line). *B*, Survival probability for 20 monkeys with *Mhc* class I allele *Mamu-A*1303* (dotted line) and 71 monkeys without *Mamu-A*1303* (solid line).

We next fitted a log linear model to the failure time data using the LIFEREG procedure as implemented in the SAS software package. A χ^2 test was performed to assess whether the factor belonging to a given allele is significantly different from zero ("no effect"). *NB5* and *Mamu-A*08* are not significant predictors of survival time if *Mamu-A*01* and *Mamu-A*1303* are also taken in account, but not vice versa. In summary, the only two independent alleles associated with longer survival detected are *Mamu-A*01* and *Mamu-A*1303*. The alleles *NB5* or *Mamu-A*08* linked to them have no measurable effect.

We next assessed whether the protective effects of *Mamu-A*01* and *Mamu-A*1303* showed a dosage effect. To this end, carriers of either allele were further distinguished according to whether or not they also carried the other allele or any of *NB2*, *Mamu-B*17*, or *Mamu-A*11*. *NB2* was included because carriers had a significantly lower viral load; therefore, *NB2* may have a protective effect. *Mamu-B*17* and *Mamu-A*11* were included because the

Table IV. *Log rank, χ^2,* p *value, and mean survival time of SIV-infected rhesus monkeys in relation to a specific rhesus monkey* Mhc *class I allele*

Mhc Allele	Log Rank	χ^2	*p*	msrvt(+)[a]	msrvt(−)[a]
*A*01*	−9.66	10.05	0.0015	75.4 ± 5.9	50.3 ± 4.1
*A*08*	−7.31	3.90	0.0482	57.4 ± 4.6	50.2 ± 5.1
*A*1303*	−9.49	8.02	0.0046	67.6 ± 6.2	50.3 ± 4.3
NB5	−10.18	9.33	0.0023	69.2 ± 5.9	49.6 ± 4.3

[a] Mean survival time (msrvt) is given in weeks post-SIV infection; (+), carriers; (−), noncarriers of the respective rhesus monkey *Mhc* class I allele.

presence of these alleles had distinguished rapid from slow progressors in a previous study (18, 42). In this study, the number of *Mamu-B*17* carriers was too small to detect a significant effect. It should be noted that the five carriers of *Mamu-B*17* had a mean survival of 66 wk postinfection (wpi). Two of them were sacrificed at weeks 57 or 70 postinfection with symptoms of AIDS. The other three animals were euthanized before the onset of clinical AIDS at week 25, 79, or 100. *Mamu-A*11* was detected only in two slowly progressing animals.

The eight monkeys carrying *Mamu-A*01* alone had a mean survival time of 70.3 ± 8.4 wpi. The five *Mamu-A*01*-positive animals with one or more of the putative protective class I alleles were all censored because they were either euthanized without AIDS or are still alive. However, three of them were characterized by a particularly long symptom-free survival time (censored after 104, 110, and 360 wk, while two of them are still alive). The difference between these two groups was statistically significant (log rank $\chi^2 = 4.1098$, 1 *df*, $p = 0.043$). A similar effect was observed for *Mamu-A*1303*. Two of the carriers of *Mamu-A*1303* alone had very early onset of AIDS at 14 wpi. However, the difference between single and multiple carriers failed to reach statistical significance (log rank $\chi^2 = 2.9621$, 1 *df*, $p = 0.085$). It should be noted that the two long-term nonprogressors had *Mamu-A*01* and *Mamu-A*1303*, whereas the third animal with both alleles was euthanized at 18 wpi without symptoms.

Figure 4 summarizes the results of this study. Fifty percent of the animals that did not carry either *Mamu-A*01* or *Mamu-A*1303*, *Mamu-B*17*, *Mamu-A*11*, or *NB2* were euthanized within 30 wk after infection with symptoms of clinical AIDS. In contrast, survival probability increased with the numbers of alleles considered to protect against a fast disease course. When the survival times of the animals that did not carry one of the "protective" alleles were stratified by the infecting virus, no significant difference was detected between the cohorts ($p = 0.64$, log rank test), excluding any effects due to potential differences in the pathogenicity of the infecting virus.

Peptide binding pockets of Mamu-A*01 *and* Mamu-A*1303

*Mamu-A*1303* belongs to an allelic lineage with members differing from each other by only a very few amino acids. Variants of *Mamu-A*13* were detected in reasonable frequencies in the monkey cohort described here. The most frequent variant was *Mamu-A*1302* not associated with longer survival. Therefore, we analyzed whether the amino acid exchanges occurred in the putative peptide binding pockets. Mamu-A*1303 and Mamu-A*01 share the same amino acids in the pockets accommodating peptide positions P1, P2, and P3. The pockets B and C which bind peptide positions P2 and P3 contribute the most to the peptide binding specificity of Mamu-A*01 (43). Interestingly, Mamu-A*1303 is distinguished from the other members of the *A*13* lineage in the pocket B accommodating P2 (Table V). At position 70, Mamu-A*1303 possesses a glutamic acid residue like Mamu-A*01, whereas the other A*13 variants contain alanine in this position. This amino acid exchange probably alters the peptide binding

Table V. *Amino acid residues of pockets C, D, and F probably determining the peptide binding specificity of rhesus monkey* Mhc *class I molecules*[a]

	C Pocket Residue No.								
Mhc Allele	9	22	70	73	74	97	99	114	116
*A*01*	Y	F	E	N	A	R	V	E	Y
*A*1303, NA4*	—	—	—	—	—	—	—	—	—
*A*1302*	—	—	A	—	—	—	—	—	—

	D Pocket Residue No.					
	99	114	155	156	159	160
*A*01*	V	E	S	M	Y	L
*A*1303, *1302, NA4*	—	—	R	—	—	—

	F Pocket Residue No.[b]	
	77	80
*A*01*	N	T
*A*1303, *1302, NA4*	A	N

[a] Predicted amino acids identical to Mamu-A*01 are indicated by dashes. Amino acid residues influencing the specific peptide binding in pocket B are identical in the class I molecules listed here.
[b] Amino acids identical in all alleles are not shown.

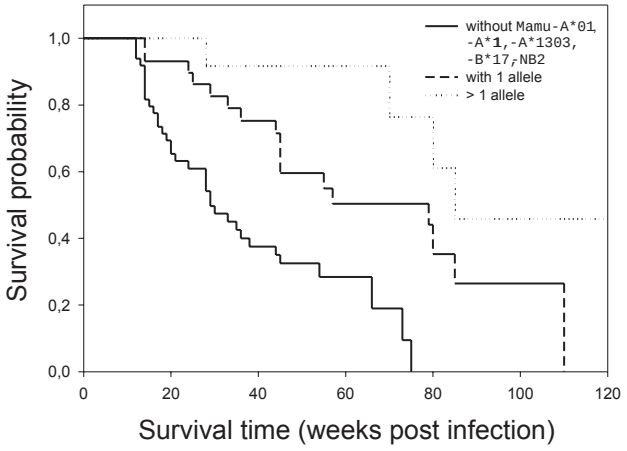

FIGURE 4. Survival probability of 91 SIV-infected rhesus monkeys stratified by the number of *Mhc* class I alleles considered to delay onset of symptoms or to be associated with lower postacute set-point viral load (*Mamu-A*01, -A*11, -A*1303, -B*17*, and *NB2)*. Survival probability curves of animals carrying no allele (solid line, $n = 49$), one allele (dashed line, $n = 29$), or more than one allele (dotted line, $n = 13$) are shown.

specificity. Mamu-A*1303 differs by a few residues from Mamu-A*01, however, in pockets D, E, and F accommodating peptide positions P4, P7, and P9. Thus, one could speculate that Mamu-A*01 and Mamu-A*1303 present partially overlapping, but not identical peptide epitopes. Since the residues in contact with the TCR are different, a larger and more diverse pool of CTLs may be stimulated when both molecules are present. Interestingly, the pockets accommodating peptide positions P2 and P3 are also identical in Mamu-A*03. Albeit it is tempting to extrapolate from amino acid sequence similarities to peptide binding properties, it should be noted that the amino acid residues of NA4 putatively involved in peptide binding are identical to Mamu-A*1303. However, *NA4* is not positively associated with disease progression. The reasons are unknown but could be related to differences in the cell surface expression level.

Discussion

One aim of the study was to establish typing techniques for rhesus macaque *Mhc* class I alleles. When the study was initiated, no molecular typing techniques had been published except for *Mamu-A*01*. In the meantime, typing techniques for two other alleles, namely, *Mamu-B*17* and *Mamu-A*02* have been described (21, 22).

Using the 15 primer pairs, at least 21 different class I alleles were detected in a cohort of 91 monkeys. It is likely that several *Mhc* alleles still remain undetected since not all PCR products have been sequenced. Using the same PCR conditions in macaques of Chinese origin, another 40 *Mhc* class I alleles have been identified by subsequent DNA sequence determination (data not shown). Since up to nine class I alleles were detected per monkey, the technique described here allows some insight into the genetic diversity of the class I genes of rhesus macaques. Thus, it represents a further step toward a more sophisticated *Mhc* class I typing technology for rhesus macaques.

For a few alleles, frequencies have also been studied by other groups. *Mamu-A*08* was found, for example, in 8 (32%) of 25 macaques (24). The average allele frequency detected here was 48%. The frequency of *Mamu-B*07* was ~50%, whereas in our monkey cohorts the frequency was ~28%. The frequency of the *A*13* variants was ~40% which is in the same range as detected here. The frequency of *Mamu-A*02* has been reported to be 18–28% depending on the monkey cohort (22). Thus, our results are within the expected range of genetic variability among rhesus macaques of Indian origin.

The relation of single alleles to the disease course of SIV-infected monkeys showed that the only class I allele influencing disease progression robustly was *Mamu-A*01*. It was significantly associated with lower set-point viral load and longer survival time. The other alleles with significant prognostic potential were *Mamu-A*1303* in terms of survival time and *NB2* with respect to viral load. In addition, increasing numbers of these alleles are associated with prolonged survival time. About 50% of the monkeys with none of these alleles or *Mamu-B*17* died within 30 wpi with symptoms of AIDS. This indicates that we probably have identified the most frequent *Mhc* class I alleles positively influencing disease course in these animals.

To date, Mamu-A*01 is the best studied rhesus macaque class I molecule. It seems to elicit an immunodominant CTL response in the acute as well as in the chronic phase of infection (44). Furthermore, a recent report has shown that *Mamu-A*01* may positively influence the results of vaccine studies (45). Detailed analysis of the SIV-specific immune response elicited by Mamu-A*1303 especially with respect to the variants containing an amino acid exchange at position 70 and NB2 will have to be performed.

*Mamu-A*01* was linked to *NB5* and *Mamu-A*1303* was strongly linked to *Mamu-A*08*. Although, statistically, neither *NB5* nor *Mamu-A*08* are associated with disease progression, they may present SIV peptides and a positive effect cannot be excluded completely. It is worthy of note that SIV-derived peptide epitopes have been reported for Mamu-A*02, and Mamu-A*08 (22, 46). However, we did not detect any significant positive associations of these alleles with disease progression although they were present in reasonable frequencies. Thirty percent of the *Mamu-A*02* carriers had to be euthanized within 28 wpi due to clinical symptoms of AIDS. The presence of *Mamu-A*02* was verified by DNA sequence analysis. Since we have recently described an *Mhc* class II genotype associated with rapid disease progression, we analyzed whether the *Mamu-A*08* or *Mamu-A*02* was present in higher frequencies in the animals possessing this genotype, and thus whether a linkage effect accounted for the lack of positive influence on the disease course. Fifteen of 31 animals euthanized with AIDS-related symptoms within 30 wpi possessed the *Mhc* class II-encoded putative rapid progressor genotypes (homozygosity for *Mamu-DQB1*0601, DRB1*309, DRB*W201* or *Mamu-DQB1*1806, *0601, DRB1*0406, DRB5*0301, DRB1*309, DRB*W201*) (19). *Mamu-A*02* was absent in all 15 animals carrying one of these genotypes. The frequency of *Mamu-A*08* was 27% in these animals and thus met the expectations. Therefore, any effect due to an overrepresentation of these alleles in animals with putatively unfavorable *Mhc* class II alleles can be excluded.

The CTL responses directed against peptides presented by Mamu-A*02 were detected in animals with a chronic infection status (22). According to our data, it seems plausible that these animals carried other *Mhc* class I alleles associated with longer survival. Vigorous CTL responses triggered by other class I molecules may have helped to contain the virus, to reduce the appearance of escape mutants, or to induce the enhanced expression of the *Mamu-A*02* by IFN-γ and other Th1-related cytokines so that immune responses mediated by *Mamu-A*02* were measurable.

The next issue to be addressed is whether the previously described *Mhc* class II-encoded "rapid progressor genotype" could be explained by a linkage effect. These class II genotypes were linked to several distinct class I haplotypes. However, except for one rapid progressing animal with *Mamu-A*1303*, none of these animals carried any of the alleles associated with longer survival.

Thus, whether the described *Mhc* class II genotypes exert a negative influence on the disease course is unclear. To date, we are aware of only two animals with this genotype which survived for longer. We hypothesized earlier that the presence of a class I molecule able to sustain strong SIV-specific CTL responses may prevent rapid disease progression in these animals. One animal (8482; Ref. 19) carried *Mamu-A*1303* and *Mamu-B*17*, whereas the other animal carried *Mamu-A*01*, *NB2*, and a rare allele similar to *Mamu-A*11*, supporting this suggestion. Unfortunately, there were no other animals with similar class I allele combinations but different *Mhc* class II alleles that could be used for comparison. However, the predictive value of this *Mhc* class II genotype in rhesus macaques originating from the Caribbean Primate Research Center is probably close to 100%.

In conclusion, the results demonstrate that *Mhc* class I genes exert a major influence on disease progression in SIV-infected macaques. *Mhc* typing can thus be used to predict the disease course. Animals likely to develop a disease course representing one of the extremes of disease variability, namely, rapid progression or long-term survival, can be identified before the onset of an experiment. Allocating untyped animals in small numbers to treated and control groups may severely confound the results of AIDS vaccine trials or treatment trials in rhesus monkeys. Given these implications, the results described here can contribute to a significant improvement of such experiments, and the studies should therefore be extended. In addition, non-MHC genes influencing viral containment must also be identified to improve the understanding and the quality of preclinical studies in rhesus monkeys.

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