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Old and New World arenaviruses share a highly conserved epitope in the fusion domain of the glycoprotein 2, which is recognized by Lassa virus-specific human CD4+ T-cell clones

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Abstract

Data from human studies and animal experiments indicate a dominant role of T-cells over antibodies in controlling acute Lassa virus infection and providing immunity to reinfection. Knowledge of the epitopes recognized by T-cells may therefore be crucial to the development of a recombinant Lassa virus vaccine. In order to study human T-cell reactivity to the most conserved structural protein of Lassa virus, the glycoprotein 2 (GP2), seven GP2-specific CD4+ T-cell clones (TCCs) were generated from the lymphocytes of a Lassa antibody positive individual. All TCC displayed high specific proliferation, showed DR-restriction, and produced IFN-γ upon stimulation with recombinant GP2. The epitope of four of the clones was localized to a short stretch of 13 amino acids located in the N-terminal part of GP2 (aa 289–301, numbering according to sequence of GPC). This epitope is conserved in all strains of Lassa virus and lymphocytic choriomeningitis virus (LCMV), shows >90% similarity in all New World arenaviruses of clade B, and overlaps with the proposed fusion domain of GP2. Peptides with conservative aa exchanges, as they naturally occur in the epitope 289–301 of the Old World arenavirus Mopeia and some New World arenaviruses, continued to effectively stimulate the Lassa-GP2-specific T-cell clones tested. The finding of a human T-helper cell epitope, which is highly conserved between Old and New World arenaviruses, is of importance for the design of arenavirus vaccines.

Keywords: Lassa virus; CD4 T-cells; Epitopes; Glycoprotein 2

Introduction

Lassa virus is endemic in certain parts of West Africa and causes an estimated 150 000 clinical cases of a systemic viral illness per year, with a mortality of 10–15% (McCormick et al., 1987). As a highly infectious hemorrhagic fever virus for which no vaccine is available, it is listed as a category A biological weapon agent (Klotz et al., 2002). Experimental immunization of monkeys or guinea pigs with recombinant vaccinia viruses or alphavirus replicons expressing Lassa virus structural proteins has been shown to protect 85–100% of animals against a lethal challenge (Fisher-Hoch et al., 2000; Pushko et al., 2001). It was also shown that expression of both glycoproteins of Lassa virus (GP1 and GP2) by the recombinant vector is necessary and sufficient for protection of nonhuman primates (Fisher-Hoch et al., 2000). In the guinea pig model of Lassa fever, adoptive spleen cell transfer of animals immunized with different Old World arenaviruses protected naive animals from homologous and heterologous infection, whereas transfer of serum did not and the protection was shown to be conferred by CD8+ cytotoxic cells (Jahrling and Peters, 1986). However, mice immunized with a recombinant

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vaccinia virus expressing the glycoprotein of Lassa virus were protected against LCMV infection exclusively by GP2-specific cytotoxic CD4+ T-cells in the absence of N antibodies (La Posta et al., 1993), and it was speculated that this type of cells could generally be involved in cross-protective immunity against heterologous arenavirus infections.

The protection induced by recombinant Lassa virus vaccines is rather short-lived and independent of neutralizing (N) antibodies, because these are detected neither after immunization nor after challenge (Fisher-Hoch et al., 2000; Pushko et al., 2001). Similar data pointing to a prominent role of T-cell immunity for the control of this disease have also come from human studies. Humans develop a very delayed and low N antibody response after natural infection with Lassa virus (Tomori et al., 1987), which seems to be strain specific. Furthermore, treatment of Lassa fever patients with human immune plasma has generally not yielded convincing results (Jahrling et al., 1985). One study reported a strong memory CD4+ T-cell response against the nucleoprotein of Lassa virus in healthy Lassa-antibody seropositive as well as seronegative persons from an endemic region, who had not been reinfected with the virus for at least 6 years (ter Meulen et al., 2000). Because any recombinant Lassa virus vaccine will have to protect against different strains of the virus, presumably on the basis of a robust T-cell immunity alone, we studied the T-cell response against the most conserved structural protein of Lassa virus and all arenaviruses, the glycoprotein 2 (GP2).

Results

Peripheral blood mononuclear cells of seven lymphocyte donors proliferate in response to both recombinant GP2 and NP of Lassa virus (JOS)

Seven individuals with statistically significant lymphocyte proliferation in response to stimulation with both recombinant NP and GP2 of Lassa virus were identified (Table 1). Three of seven (42.8%) lymphocyte donors had anti-Lassa antibodies detectable by either indirect immunofluorescence (IIF) or ELISA (Table 1).

Specific proliferation was observed both in antibody positive and antibody negative donors. From donor #28, who showed the strongest proliferation response both to GP2 and NP, the GP2-specific CD4+ T-cell clones (TCCs) were generated in this study. From the same donor, NP-specific CD4+ T-cell clones have been generated previously (ter Meulen et al., 2000).

GP2-specific T-cell clones are DR-restricted and belong to Th1-phenotype

Primary and secondary cultures of peripheral blood mononuclear cell (PBMC) from donor #28 were used to generate TCC. In several rounds of cloning, >200 TCCs were generated, 42 of which were found to react specifically with recombinant GP2. For seven of these, the epitopes were mapped, with single peptides showing proliferation indices ranging from 5.8 to 126.4 (Table 2). All TCCs tested were restricted to HLA-DR, as shown by the reduction in GP2-specific proliferation when anti-DR antibody was added to the cultures (data not shown). All TCC produced IFN-γ (range, 570–2970 pg/ml for stimulation with phytohemagglutinin [PHA]; range, 293–2776 for stimulation with GP2 and synthetic peptides), but none produced IL-4.

Table 1
Proliferative response of PBMC from Lassa virus antibody positive and negative individuals

No.	Lassa-antibody			Proliferative response to[a]				PI[b]	
	Sex/age	IIF[c]	ELISA[d]	recGP2	recNP	NEG	PHA	recGP2	recNP
28	M/45	1:20	5.3	4082	24551	54	37798	75,3	452,9
1	M/27	neg	neg	11487	14975	342	70918	33,5	43,7
11	M/65	neg	neg	3143	7456	256	68573	29,2	12,3
8	M/8	neg	2.6	10736	6838	506	74253	21,2	13,5
18	F/40	neg	2.6	3838	4646	218	38749	17,6	21,3
10	F/15	neg	neg	1408	2354	92	62481	15,3	25,6
5	M/58	neg	neg	3046	7699	386	37988	7,8	19,8

[a] Units are [^{3}H]thymidine counts per minute. NEG, negative control = cell culture medium.
[b] Proliferation index. Statistically significant proliferation (definition see results).
[c] IIF, indirect immunofluorescence.
[d] IgG–ELISA. Ratio $OD_{\text{positive control well}}/OD_{\text{negative control well}}$ at a serum dilution of 1:100.

Mapping of T-cell epitopes in the GP2 of Lassa virus (JOS) using TCC

The 21 overlapping peptides that had been synthesized were first tested for proliferation induction on TCC in four pools of five peptides each. In a second step, peptides from proliferation-inducing pools were tested individually. Three epitopes were found, which in all cases were contained in two adjacent peptides. They comprise amino acids 23–35 (*282–294*), 28–40 (*289–301*), and 135–147 (*394–406*) of GP2 (Table 2 and Fig. 1). Numbers in parentheses refer to the numbering of amino acids as in the glycoprotein precursor GPC. Of seven clones, four were found to react with the two overlapping stimulatory peptides P3 and P4 (TCC 38, TCC 53, TCC 90, TCC 139), two with peptides P18 and P19 (TCC 42, TCC 141), and one with peptides P2 and P3 (TCC 12, Table 2). Epitopes (282–294) and (289–301) partially overlap with the presumed fusion peptide of Lassa virus (Fig. 1, Glushakova et al., 1990) and epitope (394–406) partially overlaps with a T-cell epitope in C3H/HeJ (H-2^k)-mice, against which LCMV/Lassa cross-reactive cytotoxic CD4+ T-cells were generated (La Posta et al., 1993; Fig. 1).

Table 2
Screening of Lassa GP2-specific TCC[a] for reactivity to overlapping peptides spanning aa 260–427 of the GPC[b]

# aa	Peptide sequence	PI[c] for						
		TCC-12	TCC-90	TCC-38	TCC-53	TCC-139	TCC-42	TCC-141
	NEG	1.0	1.0	1.0	1.0	1.0	1.0	1.0
	recGP2	13.9	51.2	10.4	5.9	11.9	34.7	10.0
$P2_{275-294}$	PGGYCLT**RWMLIEAELKCFG**	23.5						
$P3_{282-301}$	**RWMLIEAELKCFG**NTAVAKC	52.0						
$P3_{282-301}$	RWMLIEA**ELKCFGNTAVAKC**		101.4	29.6	126.4	28.1		
$P4_{289-308}$	**ELKCFGNTAVAK**CNEKHDEE		52.3	26.2	67.5	31.1		
$P18_{387-406}$	LVSNGSY**LNETHFSDDIEQQ**						18.2	4.4
$P19_{394-413}$	**LNETHFSDDIEQQ**ADNMITE						34.3	5.8

[a] All TCC are from donor #28.
[b] Peptides were used in the assay at 1, 0.1, and 0.01 μg/ml. The two peptides containing the T-cell epitope induced proliferation at all three concentrations tested. The maximum proliferation found at 1 μg/ml is shown. The standard deviation was <10%. Amino acids contained in both stimulatory peptides are boldfaced.
[c] Proliferation indices (PI) are means of one representative assay, run in triplicate.

Alignment of the identified Lassa GP2 epitopes with sequences of other Old World and New World arenaviruses revealed a high degree of conservation for epitope (289–301) (Table 3). This epitope is 100% identical in all Lassa strains and highly conserved in all Old World arenaviruses, showing only two conserved amino acid substitutions. It is also highly conserved in New World arenaviruses of clade B and in some of clade A, which comprise the South American viruses capable of causing hemorrhagic fever in humans (Guanarito, Sabia, Junin, Machupo viruses) and North American arenaviruses (Bear Canyon and Whitewater Arroyo virus) respectively.

TCC 53 cross-reacts with epitopes located in the presumed fusion region of glycoprotein 2 of the Lassa virus (JOS) and the arenavirus Mopeia (MOP)

Comparison of the JOS epitopes with the homologous sequences in the glycoprotein 2 of the presumably nonpathogenic African arenavirus MOP is given in Fig. 2. Epitope (282–294) shows three nonconserved and one conserved amino acid substitution (77% homology), whereas both aa substitutions in epitope (289–301) are of the conserved type. These two epitopes are located in three overlapping peptides (P2, P3, and P4), which were synthe-

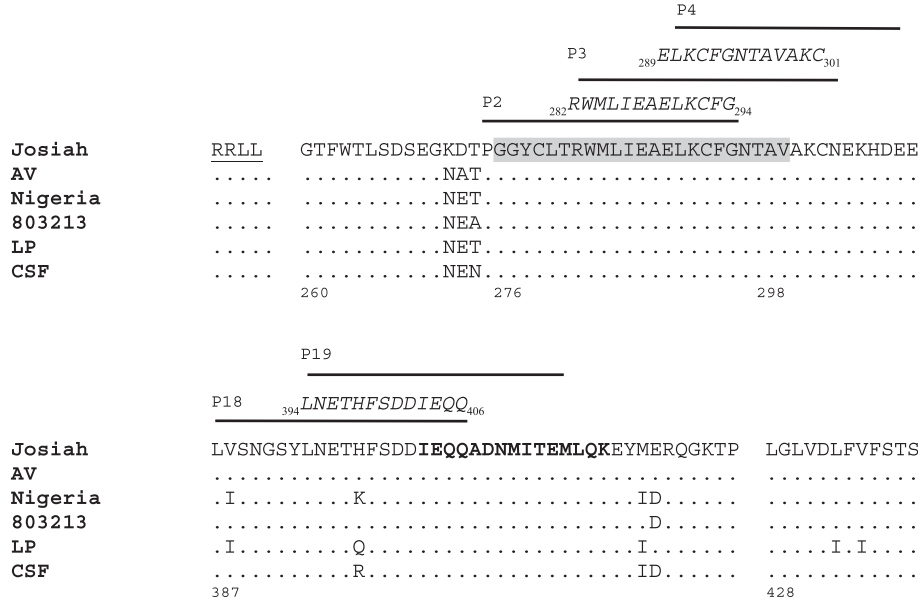

Fig. 1. Alignment of human CD4 T-cell epitopes on GP2 of Lassa virus, strain Josiah, with other Lassa virus strains. The glycoprotein precursor (GPC) cleavage motif RRLL is underlined and the presumed fusion peptide (aa 276–298, (9)) is boxed. Bold lines indicate stimulatory peptides P2, P3, P4, P18, and P19. Numbering of aa according to the sequence of GPC of Lassa virus, strain Josiah. Amino acids in italics: presumed T-cell epitopes, contained in the overlapping region of two stimulatory peptides. Boldface aa: epitope for cytolytic mouse CD4+ T-cells, cross-reactive with Lassa and LCMV (17). Start of presumed transmembrane domain of GP2 is at aa 428 (Genbank accession numbers: Josiah/J04324, AV/AF246121, Nigeria/X52400, 803213/AF181854, LP/AF181853, CSF/AF333969).

Table 3
Alignment of human CD4 epitopes (282–294; 289–301) in the presumed fusion region of Lassa virus glycoprotein 2 (GP2) with Old and New World arenaviruses

		282 294	289 301		
Lassa virus^{HF}	PGGYCLT	RWMLIEA*ELKCFG*	*ELKCFG*NTAVAKC		Old World Arenaviruses
LCMV	PGGYCLT	**KWMILAAD***LKCFG*$^{\$}$	*ELKCFG*NTAVAKC$^{\&}$		
Mopeia	PGGYCLT	R**S**MLI**GLD***LKCFG*	***D***LKCFGNTA**I**AKC		
	*******	: *:: :*****	:********:***		
				Clade	
Bear Canyon	PGGYCL**E**	**KWMLVAS***ELKCFG*	*ELKCFG*NTAVAKC	A	
Tacaribe	PGGYCL**E**	**KWMLVAS***ELKCFG*	*ELKCFG*NTA**I**AKC	B	
Whitewater Arroyo	PGGYCL**E**	**KWMLI SS***ELKCFG*	*ELKCFG*NTA**I**AKC	A	
Guanarito^{HF}	PGGYCL**E**	RWML**VAGD***LKCFG*	***D***LKCFGNTAVAKC	B	
Sabia^{HF}	PGGYCL**E**	RWML**VTSD***LKCFG*	***D***LKCFGNTA**L**AKC	B	New World Arenaviruses
Cupixi	PGGYCL**E**	**KWMLIAS***ELKCFG*	*ELKCFG*NTA**L**AKC	B	
Tamiami	PGGYCL**E**	**KWMLIAS*KLQ****CFG*	***KLQ****CFG*NTAVAKC	A	
Junin^{HF}	PGGYCL**E**	**E**WML**VAA*KM****KCFG*	***KM****KCFG*NTAVAKC	B	
Machupo^{HF}	PGGYCL**E**	**E**WMLI**AA*KM****KCFG*	***KM****KCFG*NTAVAKC	B	
Latino	PGGYCL**E**	**QWAVVWFGI***KCFD*	***GI****KCFD*NTA**M**AKC	C	
Oliveros	PGGYCL**E**	**QWAIVWA*GI****KCFG*	***GI****KCFG*NTAVAKC	C	
Allpahuayo	PGGYCL**E**	**QWAIVWA*GI****KCFD*	***GI****KCFD*N**AVM**AKC	A	
Flexal	PGGYCL**E**	**QWAVVWA*GI****KCFD*	***GI****KCFD*N**AVM**AKC	A	
Pirital	PGGYCL**E**	**QWAIVWA*GI****KCFD*	***GI****KCFD*N**SVM**AKC	A	
Parana	PGGYCL**E**	**QWALVWA*GI****KCFD*	***GI****KCFD*N**SVM**AKC	A	
Pichinde	PGGYCL**E**	QW**AIVWA*GI****KCFD*	***GI****KCFD*NT**VM**AKC	A	
	***** *	. :: ::**.	::**.*:.:***		

The overlap of the presumed CD4 epitopes (282–294) and (289–301) is underlined. Viruses marked with HF are significant human pathogens, causing hemorrhagic fever. Numbers above alignment indicate aa positions in Lassa virus, strain Josiah, GPC. "*" Denotes amino acids identical to Lassa–Josiah, ":" denotes conservative amino acid exchanges. "$" In epitope 282–294, the aa K (position 282) and L (position 286) are R and I in LCMV strains CH-5692 and CH-5871, the aa D (position 289) is conservatively mutated to E in LCMV-WE and callitrichid hepatitis virus. "&" In epitope 289–301, the amino acid E (position 298) is conservatively mutated to D in LCMV strains CH-5692 and CH-5871. Genbank accession numbers for viral sequences: Lassa–Josiah (J04324), LCMV-WE (P07399), Mopeia (M33879), Bear Canyon (AF512833), Tacaribe (NC_004293), White Water Arroyo (AF228063), Guanarito (AY129247), Sabia (U41071), Cupixi (AF512832), Tamiami (AF512828), Junin (D10072), Machupo (AY129248), Latino (AF512830), Oliveros (U34248), Allpahuayo (AY081210), Flexal (AF512831), Pirital (AF485262), Parana (AF512829), Pichinde (AF081554).

sized according to the sequence of MOP virus. The proliferation of TCC 53, the epitope of which is represented by the overlap of peptides P3 and P4, was nearly equally stimulated either by JOS-P3/P4 or MOP-P3/P4.

Proliferation of bulk PBMC in response to stimulation with peptides P2, P3, P4 and comparison with ProPred-predicted binding motifs

The PBMC from six donors were directly stimulated with single peptides. Two of the six donors (no. 8, 11) showed a specific proliferation against the single peptides P3 and P4 located in the presumed fusion region of GP2 (Fig. 3). Including the donor of the TCC (#28), three of seven persons (43%) with significant proliferation in response to the recombinant Lassa virus GP2 recognized either peptide P2, P3, or P4. The absence of a proliferative response could be due to a low frequency of specific memory CD4+ T-cells, or a major histocompatibility complex class II (MHC-II) background not allowing for presentation of the respective peptides. To this end, we performed an epitope prediction with the software ProPred, using the sequences of P2, P3, and P4 and the MHC-II alleles currently available for analysis. Table 4 shows that for those alleles for which a virtual matrix is available, there was a good correlation between experimentally observed proliferation and prediction of epitopes. Only in one case (donor #10) no significant proliferation was observed despite prediction of an epitope.

Discussion

Evidence for the importance of T-cell immunity in human Lassa virus infection is so far largely indirect. Lassa fever patients are viremic throughout the acute phase of the disease in the presence of high titered, non-neutralizing antibodies (Johnson et al., 1987) and develop low-titered neutralizing antibodies only late in convalescence, that is, 3 to 4 months after the acute phase (Tomori et al., 1987). The virus-neutralizing activity of these sera seems to be strain specific (Jahrling et al., 1985). A strong CD4+ memory T-cell response against the nucleoprotein of Lassa virus was recently shown in persons living in a Lassa-endemic region in West Africa, independent of the presence or titer of anti-Lassa antibodies and in the proven absence of a Lassa virus reinfection during the past 6 years (ter Meulen et al., 2000).

Another line of evidence for the dominance of T-cells over antibodies in Lassa virus infection comes from exper-

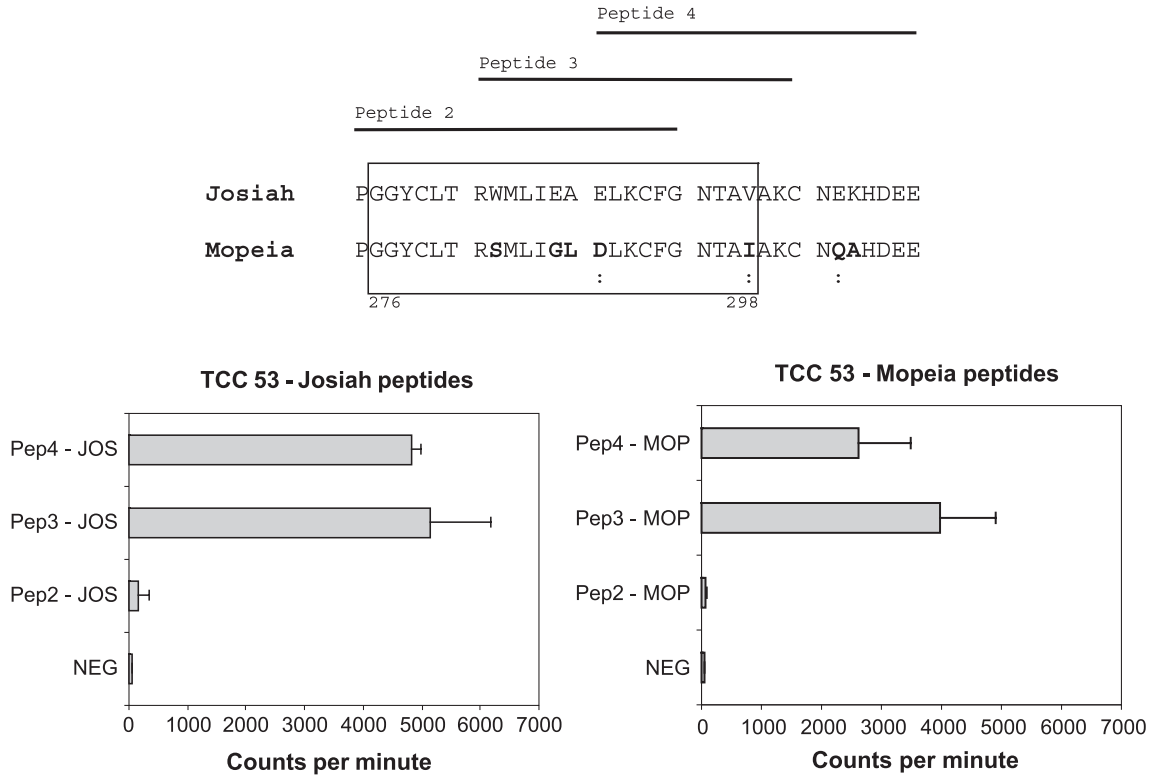

Fig. 2. Reactivity of TCC 53 with stimulatory peptides derived from the sequence of Lassa virus, strain Josiah, and of the African arenavirus Mopeia. Presumed fusion peptide (aa 276–298) is boxed. Bold lines indicate stimulatory peptides P2, P3, and P4. Numbers below alignment indicate aa positions in Josiah-GPC. Amino acids that differ between Josiah and Mopeia are boldface. ":" Denotes conservative amino acid exchanges.

imental vaccination studies. Immunization of monkeys and guinea pigs with the Lassa-related, presumably nonpathogenic East-African arenavirus Mopeia, or the Old World arenavirus lymphocytic choriomeningitis virus (LCMV) confers immunity against Lassa fever (Jahrling and Peters, 1986; Kiley et al., 1979). In the guinea pig, this protection was shown to be mediated by CD8+ cytotoxic cells, but not serum. Recombinant vaccinia viruses and VEE replicons expressing Lassa virus structural proteins (Fisher-Hoch et al., 2000; Pushko et al., 2001) have also conferred partial or full protection against disease, without neutralizing antibody activity being detectable in the sera of these animals either after immunization or after challenge. Because Lassa virus strains vary up to 15% in their amino acid sequence, the cross-protection of recombinant Lassa vaccines against heterologous strains is an important issue that has not yet been addressed in challenge experiments. However, 30% of mice immunized intragastrically with an attenuated salmonella strain expressing the Lassa virus nucleoprotein were protected against a lethal LCMV challenge (Djavani et al., 2001), and cross-reactive cytotoxic CD4+ T-cells against the LCMV-glycoprotein 2 could be induced in mice by immunizing with a recombinant vaccinia virus expressing the Lassa-glycoprotein precursor GPC (La Posta et al., 1993). It was speculated that this type of cells could generally be involved in cross-protective immunity against heterologous arenavirus infections. Taken together, the above outlined observations and experiments imply that the cellular immunity plays the major role in resolution of acute Lassa virus infection and an important role in prevention of reinfection, with strong CD8+ and CD4+ T-cell responses being induced by the virus.

We show here, that persons from an endemic area with strong CD4+ T-cell responses against the nucleoprotein of Lassa virus, strain Josiah, also display strong responses against the glycoprotein 2 (GP2), which is the most conserved protein in all Lassa virus strains (Bowen et al., 2000) and also the most conserved protein between Old and New World arenaviruses (Charrel et al., 2002; Weber and Buchmeier, 1988). As described for the nucleoprotein before, we observed T-cell reactivity against GP2 in Lassa antibody-positive and negative persons (ter Meulen et al., 2000). GP2 reactive T-cell clones generated from one lymphocyte donor showed DR-restriction and were of the Th1 phenotype, as we reported for T-cell clones reactive with the nucleoprotein of Lassa virus. The epitopes for five of the seven GP2-specific TCC were mapped to a stretch of 20 aa, spanning a hydrophobic and helical region in the N-terminal part of GP2, with four clones recognizing an epitope defined by amino acids 289–301 and another clone reactive with the overlapping

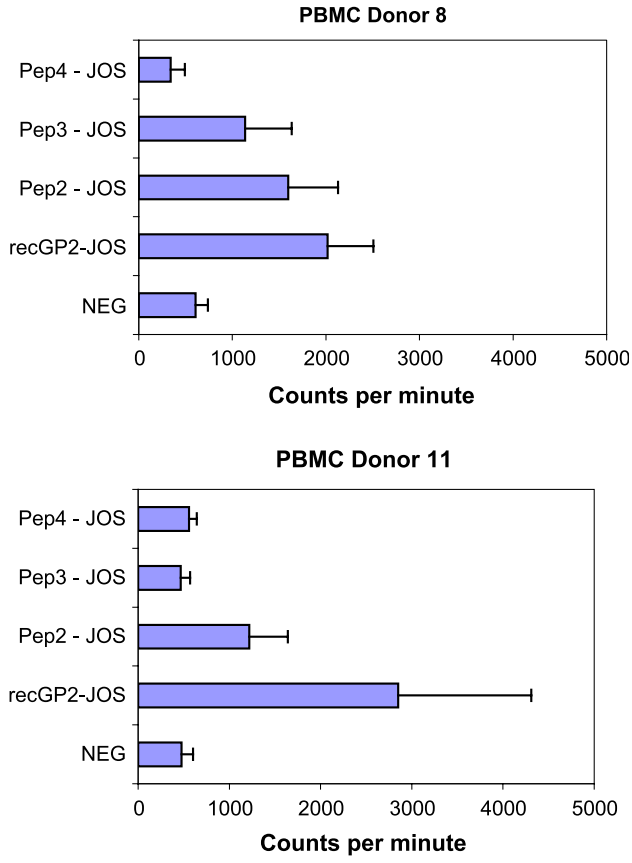

Fig. 3. Freshly isolated PBMC of two donors were stimulated with recGP2 of Lassa virus, strain Josiah, or peptides P2, P3, and P4, spanning the presumed fusion region. Assays were run in triplicate, standard error bars are shown.

epitope 282–294. Both epitopes overlap with a region that has been predicted by computer modeling and experimental data (fusion of artificial liposomes) to mediate fusion of the viral and cellular membranes during the infection process (aa 276–298, Glushakova et al., 1990). Alignment of this region reveals a high degree of homology in all arenaviruses, compared to an overall homology between Old World (OW) and New World (NW) arenaviruses of 36.5–45.7% for GPC and approximately 60% similarity for GP2 (Charrel et al., 2002). The first six amino acids of the presumed fusion peptide (PGGYCL, aa 276–281) are 100% conserved in all Old and New World arenaviruses, and overlap with a CD8+ T-cell epitope of Lymphocytic Choriomeningitis virus for H-2D^b-mice (SGVENPGGYCL, Ciatto et al., 2001). The following amino acids 282–288 are only up to 50% conserved (chemical identity), whereas aa 289–301 are 100% conserved in all Old World arenaviruses and 92% conserved between Old and New World arenaviruses of clades B and some of clade A. It thus appears that the human CD4+ T-cell epitope (289–301) is located in a region of GP2 that is most likely evolutionary highly conserved because of its functional importance. Interestingly, the third CD4+ epitope on GP2 (aa 394–406) partially overlaps with an epitope recognized by cytotoxic CD4+ T-cells of H-2^k mice (La Posta et al., 1993).

Localization of human CD4+ T-cell epitopes in conserved regions of viral proteins has been reported before. In hepatitis C virus infection, dominant, promiscuous, and highly conserved CD4+ T-cell epitopes have been identified on the nonstructural proteins NS3 and NS4 (Diepolder et al., 1997; Lamonaca et al., 1999). Dominant CD4+ epitopes were also shown to be present in highly conserved, functional domains (transcription activation domain) of the Human Papillomavirus E2 protein (de Jong et al., 2002), in conserved regions of the gag and pol proteins of HIV-1 (van der Burg et al., 1999; Wilson et al., 2001), the VP4 and VP2 proteins of Coxsackievirus B4 (Marttila et al., 2002), the adenovirus capsid protein hexon (Olive et al., 2002), and the NS3 of Dengue virus. In the latter, CD4+ clones cross-reactive between different serotypes were also proven to be cytotoxic (Kurane et al., 1998). It thus appears

Table 4
Reactivity of PBMC to stimulatory peptides spanning the presumed fusion peptide of Lassa virus GP2, in comparison with MHC-II-based epitope prediction (TEPITOPE analysis)

Donor[a]	MHC-II (DR)[b]			P2	P3	P4[c]	TEPITOPE prediction[d]
28[e]	DRB1*0101[f], *0301	*DRB3*0301*[g]		TCC	TCC	TCC	WMLIEAELK
1	DRB1*0102, *1303*	*DRB3*0101*		neg.	neg.	neg.	no epitope
11	DRB1*1102, *1503*	DRB3*0301	DRB5*0101	2,6	neg.	neg.	WMLIEAELK
8	DRB1*1101, *–	*DRB3*02XX*		2,6	1,9	neg.	WMLIEAELK, FGNTAVAKC
18	DRB1*0102, *1303*	*DRB3*0101*		neg.	neg.	neg.	no epitope
10	DRB1*0901, *1503*	*DRB4*01XX*	DRB5*0101	neg.	neg.	1,5	WMLIEAELK
5	DRB1*1301, *–	*DRB3*0101*	*DRB3*02XX*	neg.	1,5	neg.	no epitope

[a] Numbering of lymphocyte donors as in Table 1.
[b] Because all GP2-specific TCC are DR-restricted, only epitopes for DR-alleles were computed.
[c] Proliferation index of PBMC stimulated with peptides P2 (PGGYCLTRWMLIEAELKCFG), P3 (RWMLIEAELKCFGNTAVAKC) or P4 (ELKCFGNTAVAKCNEKHDEE). All assays run in triplicate, a P.I. ≥ 2 is considered significant.
[d] Predictions of optimally binding peptides within the postulated fusion domain of GP2 of Lassa virus, strain Josiah.
[e] Donor No. 28 served as donor for the generation of P2, P3and P4 specific TCC (see Table 1).
[f] Underlined alleles: MHC-II epitopes predicted by TEPITOPE.
[g] Allels in italics: No TEPITOPE prediction possible for these alleles.

that focusing of the human CD4+ T-cell immune response on epitopes of viral proteins that are highly conserved because of their functional importance is a common theme. Obvious advantages could be cross-protection against different viral variants and strains, which might vary considerably in other parts of their proteins, as well as possibly avoiding the development of CD4+ T-cell escape mutants in persistent infections such as Hepatitis C (Wang and Eckels, 1999).

Dominant CD4 T-cell epitopes located in conserved regions of viral proteins are often also promiscuous epitopes, being presented by a wide range of different MHC-II molecules (Diepolder et al., 1997; Lamonaca et al., 1999; Olive et al., 2002; van der Burg et al., 1999; Wilson et al., 2001). To this end, we stimulated PBMC from six MHC class-II-typed donors directly with the three peptides comprising the CD4+ T-cell epitopes in the fusion region of GP2. Experimental data were compared to computer-generated prediction of epitopes for the given HLA alleles. There was a good correlation with epitope prediction and only two of the donors showed a significant proliferation. Their HLA-DR alleles predicted to present the peptides were DRB1*1101 and DRB5*0101, compared to DRB1*0101 of the donor from which the epitope defining TCC were derived. In this small series, we did therefore not observe very promiscuous binding of the epitopes; however, the assay might be too insensitive too pick up a low number of memory T-cells. The frequency of the above alleles has been estimated at 23%, 17%, and 13% for the population of African descent and 17%, 24%, and 20% for the Caucasian population, respectively (Sanchez-Mazas, 2001; Zachary et al., 2001).

The degree of conservation of immunodominant T-cell epitopes or MHC binding regions between different virus strains will be pivotal for cross-protective immunity in a predominantly T-cell controlled infection such as Lassa fever. Because expression of both glycoproteins GP1 and GP2 from recombinant vectors is necessary and sufficient for protection of nonhuman primates against Lassa virus challenge, our finding of highly conserved CD4+ T-cell epitopes in GP2 provides first evidence that a recombinant vaccine may offer cross-protection on the basis of a T-cell response. Furthermore, the identified CD4 epitopes are also highly conserved in clade B of the New World arenaviruses, which contain the South American viruses causing hemorrhagic fever (Guanarito, Sabia, Junin, and Machupo). Interestingly, a highly conserved epitope for monoclonal antibodies cross-reactive with Old and New World arenaviruses is also located on GP2 (Weber and Buchmeier, 1988; Ruo et al., 1991). Further analysis of conserved T-cell epitopes on arenavirus structural and nonstructural proteins might lead to the design of an optimal recombinant vaccine, which offers protection against all Old and New World arenaviruses causing hemorrhagic fever in humans.

Materials and methods

Study population

Individuals with a past exposure to Lassa virus were identified during epidemiologic investigations in the Republic of Guinea, West Africa, based on seropositivity or T-cell reactivity with the nucleoprotein of Lassa virus (ter Meulen et al., 2000). After obtaining informed consent, blood samples were drawn for serology and preparation of peripheral blood mononuclear cells (PBMCs). Seven persons were chosen for this study, because their PBMC strongly proliferated in response to stimulation with two recombinant Lassa virus proteins NP and GP2. The donor of the PBMC from which the GP2-specific T-cell clones (TCC) were derived serves as a positive control in all T-cell assays since 1997.

Detection of Lassa virus antibodies with indirect immunofluorescence and ELISA

In the BSL4 facility of the University of Marburg, Lassa virus (Josiah strain, JOS), was grown in Vero cells (ATCC CCL 81), propagated in DMEM medium supplemented with 5% fetal calf serum. For indirect immunofluorescence, a standard procedure was performed as described previously (ter Meulen et al., 1998). To detect low-titer antibodies that might be missed in IIF when testing sera at a dilution of 1:20 or higher, an ELISA was also employed. Lassa virus was grown in several large flasks (75 cm^2) for up to 1 week, and the supernatant was cleared from floating cells by centrifugation at 1000 × *g* for 30 min. It was then pelleted through a 10-ml sucrose (20% w/v) cushion in an SW28 rotor at 20.000 RPM for 2 h. The pellets were resuspended in TNE buffer and subjected to 30 kGy of Co60 γ-irradiation for inactivation, which was tested by two blind passages of the inactivated material in cell culture. The protein content of the inactivated virus solution was adjusted to 100 μg/ml and stored at −80 °C until further use. NUNC-Maxisorp plates were coated with a diluted virus solution of 10 μg/ml overnight at 4 °C, washed once with PBS/Tween 0.5% and blocked with PBS/5% skimmed milk powder for 1 h. For each serum, a negative control well was coated with uninfected Vero-cell supernatant, cleared of floating cells. Human sera were diluted (1:100) in PBS/5% skimmed milk and pipetted onto the plate. After an incubation of 1 h at room temperature and six washes (PBS/0.5% Tween20), conjugate (goat-anti-huIgG-HRP, DIANOVA) was added in a dilution of 1:800 for 1 h; after six washes, the substrate (ABTS, KPL, MD, USA) was added and the plate incubated for 30 min at 37 °C. Optical densities (OD) were read after 30 min at 405 nm. Sera were regarded positive if the OD ratio of the virus-coated over the uninfected supernatant-coated well was ≥2 (ratio A), and ≥2 times the OD of the negative control serum (ratio B). The numerical value of ratio A is

given in Table 1. ELISA assays were run in duplicate wells.

Cloning, expression, and purification of a C-terminally truncated recombinant Lassa virus (JOS) glycoprotein 2 (recGP2)

The glycoprotein 2 (GP2) of arenaviruses is a type-I transmembrane protein, which is generated in infected cells through cleavage of the glycoprotein precursor GPC by the cellular subtilase SKI-1/S1P (Lenz et al., 2001). To improve its recombinant expression and solubilization in *E. coli*, a C-terminally truncated form of the gene of GP2 (encoding for the luminal domain) was reverse transcribed and PCR-amplified from Lassa virus RNA extracted from infected Vero cell culture supernatant. The PCR fragment was cloned via restriction-site containing primers (*Bam*HI, *Hin*dIII) in the procaryotic expression vector pQE30 (Qiagen, Hilden, Germany), resulting in expression of aa 1–168 of GP2 (corresponding to aa 260–427 of the glycoprotein precursor GPC). The abundantly overexpressed protein was extracted from insoluble inclusion bodies with 8 M urea and purified by Ni-chelate affinity chromatography (The QIAexpressionist, 1992). After purification (>99% as estimated from Coomassie stained SDS-PAGE gels), the protein was dialyzed against phosphate-buffered saline for 48 h at 4 °C. The concentration was determined photometrically and adjusted to 10 μg/ml. The protein solution was passed through a 0.2-μm filter and stored in 1.0-ml aliquots at −70 °C until further use. The cloning, expression, and purification of the recombinant nucleoprotein of Lassa virus has been described elsewhere (ter Meulen et al., 1998).

Synthesis of overlapping peptides comprising aa 9–168 of the Lassa virus glycoprotein GP2, strain Josiah

For T-cell epitope mapping, a set of twenty-one 20-mer peptides with 13 amino acids overlap was designed (spanning the sequence of the Lassa virus GP2 from amino acid 9–168) and synthesized using pin technology (Mimotopes Pty Ltd, Clayton, Australia).

Major histocompatibility complex class II typing of PBMC-donors

HLA class II analysis was performed after extraction of DNA with phenol/chloroform from PBMCs. Amplification of the HLA class II exons for DRB1, DRB3, DRB4, DRB5, DQB1, DQA1, and DPB1 loci was performed by PCR as described with locus-specific biotinylated primers as described previously (Erlich et al., 1991; ter Meulen et al., 2000). MHC class II alleles were determined as Donor #28: DRB1*0101,0301. DRB3*0301. DQA1*0101,0101. DQB1*02,0501. Donor #1: DRB1*0102,1303. DRB3*0101. DQA1*0101,05. DQB1*0301,0501. DPB1*0201,2601. Donor #11: DRB1*1102,1503. DRB3*0301. DRB5*0101. DQA1*0102,05. DQB1*0301,0602. DPB1*0101,*0402. Donor #8: DRB1*1101,. DRB3*02XX. DQA1*0102,05. DQB1*0301,0609. DPB1*0101,–. Donor #18: DRB1*0102,1303. DRB3*0101. DQA1*0101,05. DQB1*0301,0501. DPB1*0201,2601. Donor #10: DRB1*0901,1503. DRB4*01XX. DRB*0101. DQA1*0102,03. DQB1*02XX,0602. DPB1*0101,–. Donor #5: DRB1*1301,–. DRB3*0101, 02XX. DQB1*0301,0603. DPB1*0201,1701.

Proliferation of PBMC and generation of GP2-specific T-cell lines and clones

Procedures for the generation of nucleoprotein-specific T-cell clones have previously been described (ter Meulen et al., 2000). Briefly, 10^5 PBMC were stimulated with recombinant GP2 (10 μg/ml each), or with phytohemagglutinin (PHA, 2 μg/ml), and [^{3}H]thymidine incorporation was measured by liquid scintillation spectrometry. In parallel to proliferation assays, cultures showing proliferation microscopically were further stimulated with antigen and 5 days thereafter propagated by supplementation with 10 U/ml IL-2. For secondary stimulation with GP2, 5×10^4 cells of the primary T-cell lines were used. MHC II haplotype-matched, γ-irradiated PBMC (1×10^5) from healthy European donors served as antigen-presenting cells. To test whether GP2 was presented to TCC by DR or DQ, supernatants of the hybridomas L243 (anti-DR, Lampson and Levy, 1980) or Tu22 and Tu169 (anti-DQ, Pawelec et al., 1985) were added during proliferation assays, respectively.

In addition, PBMCs freshly obtained from six of the West African donors were stimulated directly with three single peptides (P2, P3, and P4) that span the presumed fusion peptide of GP2, at a concentration of 1 μg per individual peptide as previously described (ter Meulen et al., 2000).

The ratio of the mean proliferative response to the recombinant proteins over the negative control is given as proliferative index (PI). The proliferation was regarded as specific if the response to the recombinant proteins (or peptides) exceeded that of the negative control plus three times the standard deviation. Proliferation assays were run in triplicate unless otherwise indicated.

T-cell epitope mapping with TCC using overlapping synthetic peptides

Synthetic peptides (1 mg) were dissolved in 10 μl of DMSO and brought to a final volume of 1 ml to create a stock solution of 1 mg/ml. For cell culture, this stock solution was further diluted in RPMI to end concentrations of 10–0.001 μg/ml. At these dilutions, the traces of DMSO were found to influence neither proliferation nor cytokine production, as pretested in mixing assays (data not shown).

Cytokine assays

Measurement of cytokines in cell culture supernatants was performed as previously described (ter Meulen et al., 2000). Briefly, supernatants from cultures parallel to those used for the determination of proliferation were harvested after 3 days, stored at −20 °C, and cytokines were quantified using cytokine-specific ELISA. Recombinant human cytokines were used as reference standards (IL-4 and IFN-γ from Pharmingen, Hamburg, Germany) and the following mAb pairs were used for capture and detection: IFN-γ: NIB42/biotinylated 4S.B3; IL-4: IL4–1/biotinylated MP4-25D2 (all mAbs from Pharmingen). The working sensitivity of all ELISA assays was shown to be 50 pg/ml.

Prediction of CD4 T-cell epitopes for individual MHC-II alleles

For MHC class-II binding peptide prediction, the graphical web tool "ProPred" was used (Singh and Raghava, 2001). This algorithm employs virtual MHC-II matrices based on pocket profiles (TEPITOPE, Sturniolo et al., 1999), which provide a detailed model in which the contribution to binding of each amino acid (ligand) with each pocket/position (HLA binding cleft) is quantified. Virtual matrices are formed by assigning and combining pocket-specific quantitative binding values derived from one HLA allele to other alleles via HLA sequence comparison and extrapolating from experimental data, thereby enabling prediction of peptide ligands for a broad range of MHC-II alleles (promiscuous peptides). Presently prediction of peptide binders is possible for 51 MHC-II alleles (http://www.imtech.res.in/raghava/propred). For our analysis, the standard settings of the program were used (threshold 3%).

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