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Characterization of Human CD4$^+$ T-Cell Clones Recognizing Conserved and Variable Epitopes of the Lassa Virus Nucleoprotein

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T cells must play the major role in controlling acute human Lassa virus infection, because patients recover from acute Lassa fever in the absence of a measurable neutralizing antibody response. T cells alone seem to protect animals from a lethal Lassa virus challenge, because after experimental vaccination no neutralizing antibodies are detectable. In order to study human T-cell reactivity to single Lassa virus proteins, the nucleoprotein (NP) of Lassa virus, strain Josiah, was cloned, expressed in *Escherichia coli*, and affinity purified. Peripheral blood mononuclear cells (PBMC) obtained from 8 of 13 healthy, Lassa virus antibody-positive individuals living in the Republic of Guinea, western Africa, were found to proliferate in response to the recombinant protein (proliferation index ≥10). PBMC obtained from one individual with a particularly high proliferative response were used to generate 50 NP-specific T-cell clones (TCC). For six of these the epitopes were mapped with overlapping synthetic peptides derived from the sequence of the NP. These CD4$^+$ TCC displayed high specific proliferation and produced mainly gamma interferon upon stimulation with NP. Because variation of up to 15% in the amino acid sequences of the structural proteins of naturally occurring Lassa virus variants has been observed, the reactivity of the TCC with peptides derived from the homologous epitopes of the Nigeria strain of Lassa virus and of the eastern Africa arenavirus Mopeia was tested. With the Nigeria strain of Lassa virus the levels of homology were 100% for two of these epitopes and 85% for three of them, whereas homology with the respective Mopeia epitopes ranged from 92 to 69%. Reactivity of the TCC with peptides derived from the variable epitopes of the Nigeria strain and of Mopeia was reduced or completely abolished. This report shows for the first time that seropositive individuals from areas of endemicity have very strong memory CD4$^+$ T-cell responses against the NP of Lassa virus, which are partly strain specific and partly cross-reactive with other Lassa virus strains. Our findings may have important implications for the strategy of designing recombinant vaccines against this mainly T-cell-controlled human arenavirus infection.

Lassa virus is a negative-strand RNA virus belonging to the family *Arenaviridae*. It is the causative agent of Lassa fever, a reemerging viral hemorrhagic fever, which accounts for significant human morbidity in regions of endemicity in western Africa, with approximately 300,000 to 500,000 infections occurring each year (20). An estimated 30% of infections are symptomatic, and the clinical picture ranges from flu-like illness to fulminant hemorrhagic fever with an overall mortality of 10 to 15% (19). Humans recover from acute Lassa fever in the absence of a measurable neutralizing (N) antibody response (12). Low-titer N antibodies develop only late in convalescence (9, 28) and preferentially neutralize Lassa virus strains isolated in the same geographical region (9). Their role in protection from reinfection is presently not clear. Lassa virus reinfections presumably occur without clinically overt disease, as suggested by prospective seroprevalence studies in areas of endemicity (20). However, more clinical and virological data on this issue are needed. Attempts to vaccinate against Lassa fever in animal models, including nonhuman primates, have revealed that eliciting a strong cellular immune response protects from clinical disease, but not from infection, in the absence of measurable N antibodies (reviewed in reference 4). Even after challenge the animals developed only a very low-titer N antibody response. It was shown in the guinea pig model that infection with heterologous arenaviruses of low pathogenicity confers protection against challenge with highly virulent Lassa virus strains (10). This immunity could be passed on to naive animals only by syngeneic transfer of CD8$^+$ cytotoxic T lymphocytes (CTLs) obtained in the early phase of convalescence. Evidence for an important role for CD4$^+$ cells in protection comes from an experiment in which vaccination of mice with recombinant vaccinia virus-Lassa virus constructs elicited a response consisting of CD4$^+$ CTLs, which protected the animals from infection with lymphocytic choriomeningitis virus (LCMV), in the absence of N antibodies (16). The role of Lassa virus-specific CD4$^+$ and CD8$^+$ cells has to date not been evaluated in human disease. As cellular immunity is associated with recovery in the natural course of Lassa fever and is essential in the setting of experimental vaccines, we were interested in investigating T-cell responses of persons from areas of endemicity who were exposed to the virus. Because the struc-

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tural proteins from Lassa virus strains differ by up to 10 to 15% in their amino acid sequences, with only the strains Josiah (JOS) and Nigeria (NIG) having been sequenced completely to date, we placed a special emphasis on the question of whether naturally acquired immunity was cross-protective against different Lassa virus strains.

MATERIALS AND METHODS

Study population. Subjects for the study were identified in the Republic of Guinea, western Africa, during a population-based seroprevalence survey carried out in 1993, on the basis of antibody titers to Lassa virus of ≥1:160 as measured by indirect immunofluorescence (IIF) (26). Despite these high antibody titers, which indicate rather recent infection (19, 28), all subjects were clinically asymptomatic while being studied in 1993. They lived as peasants in villages where Lassa fever is endemic and were clinically and serologically reexamined in 1997 and 1999, with blood samples being drawn for serology in 1997 and peripheral blood mononuclear cells (PBMC) being sampled in 1999. Informed consent was obtained on all occasions. The donor of the PBMC from which the T-cell clones TCC were derived (referred to as the TCC donor) works as head of the laboratory in a regional hospital and served as regular PBMC donor and positive control in all assays since 1997.

Detection of Lassa virus antibodies with IIF. In our BSL4 facility in Hamburg, Germany, Lassa virus (JOS strain) was grown in U937 (ATCC CRL 1593) or Vero cells (ATCC CCL 81) and propagated in RPMI 1640 or minimum essential medium supplemented with 5% fetal calf serum. After approximately 1 week, the cells were harvested, spread on immunofluorescence slides, air-dried, and fixed for 1 h at room temperature. Successful infection (30 to 60% of cells infected) was shown by immunofluorescence with monoclonal antibodies raised against the nucleoprotein (NP) of Lassa virus (8). The slides were stored at −70°C until use. All sera were tested at a dilution of 1:20 in phosphate-buffered saline (PBS) by IIF, using the second antibody (goat anti-human immunoglobulin G–fluorescein isothiocyanate; Dianova, Hamburg, Germany) at a dilution of 1:50. Positive sera were then serially end point diluted in PBS.

Cloning, expression, and purification of the N-terminally truncated Lassa virus recNP. The cloning and expression of a truncated recombinant nucleoprotein (recNP) has been described elsewhere (27). Briefly, fragments of the NP gene of the Lassa virus (JOS) were amplified by reverse transcription-PCR with restriction sites for *Bam*HI and *Hin*dIII incorporated into the 5′-end and 3′-end PCR primers, respectively. Fragments of different lengths were then cloned into a T7 polymerase-driven expression vector, which adds an N-terminal tag of 10 histidine residues to the recombinant protein. Expression was performed in *Escherichia coli* BL21(DE3). Neither the whole NP nor the N terminus (amino acids [aa] 1 to 139) could be expressed, but a truncated protein (aa 141 to 569) was abundantly overexpressed, extracted from insoluble inclusion bodies with 8 M urea, and purified by nickel chelate chromatography (QIAexpressionist; Qiagen, Hilden, Germany). After purification (>99% as estimated from Coomassie-stained sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels), the protein was dialyzed against PBS at 4°C for 24 h. The concentration was determined photometrically and adjusted to 10 μg/ml. The protein solution was passed through a 0.2-μm-pore-size filter and stored in 1.8-ml aliquots at −70°C until further use.

Expression and purification of control protein recDHFR. Dihydrofolate reductase (DHFR) cloned in the T7 expression vector pQE30 (Qiagen) was expressed in *E. coli* BL21(DE3) and affinity purified using the same protocols as those for the Lassa recNP. The recombinant protein (recDHFR) was stored at a concentration of 10 μg/ml at −70°C until further use as a negative control in the proliferation assays.

Synthesis of overlapping peptides comprising aa 141 to 569 of the Lassa virus NP, strain JOS. For T-cell epitope mapping, a set of 60 20-mer peptides with 13-aa overlap was designed based on the sequence of the Lassa virus (JOS) NP and synthesized using pin technology (Abimed, Langenfeld, Germany) (5). The purity of the peptides (delivered, >70%, according to the manufacturer's specifications) was determined by analytical reverse-phase high-pressure liquid chromatography (model 172A; Applied Biosystems, Weiterstadt, Germany) using an Aquapore OD-300 column (30 by 2.1 mm; Brownlee/Applied Biosystems), as described previously (5, 17). Some stimulatory peptides (P34 homologues NIG and MOP) were further purified by HPLC and subjected to sequence analysis using an Applied Biosystems model 473A protein sequencer (5, 17).

Major histocompatibility complex class II (MHC-II) typing of PBMC donors. HLA class II analysis was performed after extraction of DNA with phenol-chloroform from PBMC. Amplification of the HLA class II exons for DRB1, DRB3, DRB4, DRB5, DQB1, DQA1, and DPB1 loci was performed as described previously by PCR with locus-specific biotinylated primers (6). The amplification products were hybridized to oligo(dT)-tailed sequence-specific oligonucleotides, which were fixed to nylon membrane strips by UV light (reverse hybridization). Hybridized amplificates were detected by incubation with streptavidin-peroxidase and dimethylbenzidine. Unambiguous alleles were assigned according to hybridization patterns and second amplification with group-specific primers.

Proliferation of PBMC and generation of T-cell lines. PBMC were separated from heparinized venous blood by gradient centrifugation on Ficoll-Paque (Pharmacia, Freiburg, Germany). Cells were adjusted to a density of 10^6/ml in RPMI 1640 supplemented with 2 mM L-glutamine, 50 μM gentamicin, and 10% heat-inactivated human AB serum. For T-cell proliferation 10^5 PBMC were stimulated with recNP (10 μg/ml), with recDHFR as the vector control (10 μg/ml), or with phytohemagglutinin (PHA; 2 μg/ml) in a total volume of 200 μl and were cultured for 4 days in 96-well round-bottom microtiter plates. Cultures were pulsed with 0.2 μCi for the last 18 h, 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 were propagated by supplementation with 10 U of interleukin-2 (IL-2)/ml. For secondary stimulation with NP, 5×10^4 cells of the primary T-cell lines were used. MHC-II haplotype-matched, γ-irradiated PBMC (10^5) from healthy European donors served as antigen-presenting cells. PBMC obtained from 11 donors were stimulated directly with 10 peptide pools containing six overlapping, synthetic peptides each, at a concentration of 1 μg per individual peptide. Cells were stimulated for 3 days and then pulsed with [^{3}H]thymidine. In the beginning of the experiments, a few proliferation assays were run with both cell culture medium and recDHFR as negative controls, including those for the generation of TCC from the TCC donor. Because we never observed proliferation in response to recDHFR, the remainder of the tests were run with cell culture medium or recDHFR as the negative control.

Generation of TCC. T cells from those lines which showed a recNP-specific proliferation were cloned at a density of 10, 3, 1, and 0.3 cells/well in Terasaki plates (Nunc, Wiesbaden, Germany) together with irradiated (4,000 rads) feeder cells (1.2×10^4), PHA (2 μg/ml), and IL-2 (Eurocetus, Amsterdam, The Netherlands; 100 U/ml). Growing TCC were picked from plates with less than 10% positive wells and restimulated in 96-well round-bottom microtiter plates at 10- to 14-day intervals. The specificity of TCC was determined by testing their proliferation in response to recNP and synthetic NP peptides (10 to 0.1 μg/ml), using 3×10^4 T cells as responders and 10^5 MHC-II haplotype-matched PBMC as antigen-presenting cells.

To test whether recNP was presented to TCC by DR or DQ, 50 μl of culture supernatant of the hybridomas L243 (anti-DR) (15) or Tu22 and Tul69 (anti-DQ) (24), respectively, was added during proliferation assays. At these concentrations the monoclonal antibodies (MAbs) are known to reduce either DR- or DQ-restricted antigen-specific proliferation of T cells by more than 90% (5); this was again pretested in our study (data not shown).

T-cell epitope mapping with TCC using overlapping synthetic peptides. Synthetic peptides (1 mg) were dissolved in 10 μl of dimethyl sulfoxide (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 1640 to final concentrations of 10 to 0.001 μg/ml. At these dilutions the traces of DMSO were found to influence neither proliferation nor cytokine production, as indicated by pretest mixing assays (data not shown).

Cytokine assays. Supernatants from cultures parallel to those used for the determination of proliferation were harvested after 3 days and stored at −20°C, and cytokines were quantified using a cytokine-specific enzyme-linked immunosorbent assay (ELISA). Two recombinant human cytokines were used as reference standards: IL-4 and gamma interferon (IFN-γ; Pharmingen, Hamburg, Germany). For cytokine ELISA the following MAb pairs were used for capture and detection: NIB42 and biotinylated 4S.B3 (IFN-γ) and IL4-1 and biotinylated MP4-25D2 (IL-4) (all MAbs were from Pharmingen) F96 Maxisorp immunoplates (Nunc) were coated with 50 μl of capture antibody (1 μg/ml for IL-4, 5 μg/ml for IFN-γ) in 0.1 M $NaHCO_2$-Na_2CO_3 buffer (pH 9.6) overnight at 4°C. After being blocked with 1% bovine serum albumin (BSA) plates were washed with PBS–0.05% Tween 20 and incubated overnight at 4°C with 50 μl of culture supernatant or cytokine at a standard dilution. Biotinylated detection antibodies were used at 0.5 μg/ml in PBS–Tween–0.1% BSA. Plates were developed after incubation with streptavidin-peroxidase complex (1:10,000; Boehringer, Mannheim, Germany), using 100 μl of tetramethylbenzidine (TMB; Roth, Karlsruhe, Germany; dissolved at 6 mg/ml in DMSO)/well as the substrate. Substrate reactions were stopped with 25 μl of 4 N H_2SO_4/well and measured at 450 nm. The working sensitivity of all ELISAs was shown to be 50 pg/ml.

RESULTS

Serology. Results of IIF tests for Lassa virus antibodies are given in Table 1. A constant decline in antibody titer was noticed for all subjects except for 8.1.10, who showed a more than fourfold rise between 1997 and 1999 and thus probably was reinfected, and the TCC donor, whose antibody titer remained constantly low at 1:20 throughout the observation period. Two individuals (10.3.35 and 2.4.31) had lost their antibodies by 1999.

PBMC from Lassa virus antibody-positive and -negative individuals proliferate in response to recNP of Lassa virus, strain JOS. The ratio of the mean proliferative response to

TABLE 1. Proliferative response of PBMC from Lassa virus antibody-positive and -negative individuals

Subject	Sex/age[a] (yr)	Lassa antibody titer[b] in:			Proliferative response[c] in 1999 to:			PI[d]
		1993	1997	1999	JOS recNP	PHA	NEG	
TCC donor	M/45	1:20	1:20	1:20	8,200	14,000	92	91*
5.1.6	M/23	1:1,280	1:160	1:160	1,390	21,200	40	35*
10.2.22	F/81	1:2,560	1:20	1:20	4,670	25,100	150	31*
5.3.23	M/24	1:1,280	1:160	1:160	2,680	28,500	90	30*
8.2.24	F/44	1:1,280	1:640	1:160	4,060	24,600	170	24*
12.2.19	M/30	1:320	1:160	1:80	3,370	24,900	160	21*
10.3.35	M/15	1:640	1:20	neg.	1,340	28,900	100	13*
8.1.10	M/16	1:320	1:20	1:160	2,960	31,200	300	10*
1.2.19	F/62	1:1,280	1:320	1:80	300	6,700	60	5
9.2.15	F/19	1:320	1:160	1:160	320	34,000	120	2.7
2.2.11	M/38	1:1,280	1:320	1:160	150	19,600	60	2.5
2.4.31	M/76	1:1,280	1:80	neg.	290	30,600	120	2.4
13.5.27	M/86	1:160	n.d.	1:40	179	9,473	79	2.3

[a] Age for persons older than 50 years is an estimate with an accuracy of ±5 years.
[b] As measured by IIF. neg., negligible; n.d., not determined.
[c] Units are [^{3}H]thymidine counts per minute. NEG, negative control, either recDHFR or cell culture medium.
[d] *, statistically significant (see Materials and Methods).

recNP (or peptide pools) to that of the negative control is given as the proliferative index (PI). The proliferation was regarded as specific if the response to the recNP (or peptides) exceeded that to the negative control plus three times the standard deviation. Proliferation assays were run in triplicate unless otherwise indicated, and the mean PI value is given. PBMC from 7 of the 11 individuals (63.6%) who tested seropositive for Lassa antibodies in 1999 showed specific proliferation to the Lassa recNP (Table 1). Of the two individuals without antibodies in 1999, one showed significant proliferation.

PBMC from Lassa virus antibody-positive and -negative individuals proliferate in response to pools of synthetic, overlapping peptides, spanning the recNP of Lassa virus, strain JOS. PBMC from 3 of 11 donors (27.3%) showed a specific proliferation (defined above) against peptide pools containing six overlapping, stimulatory peptides each (Fig. 1). The PBMC of the TCC donor from which the TCC were generated showed a PI >4 for three of five peptide pools containing the specific peptides on which the TCC epitopes could later be located (Table 2).

NP-specific TCC belong to Th1 or Th0 phenotype. Primary and secondary cultures of PBMC from the TCC donor were used to generate TCC. In several rounds of cloning, >500 TCC were generated; approximately 10% of those were found to react specifically with recNP (PIs ranging from 7 to 450). A representative clone is shown in Fig. 2A. All six NP-specific TCC, the epitopes of which were mapped (Table 2), were found to be $CD4^+$ and $CD8^-$ as determined by fluorescence-activated cell sorter (data not shown). All of them produced large amounts of IFN-γ (range, 1,400 to 12,700 pg/ml), and one was found to also produce large amounts of IL-4 (TCC 141 in Fig. 2B and C). Four clones additionally produced low levels of IL-4 (200 to 300 pg/ml), the secretion of which was not increased by specific stimulation with recNP. However, no IL-4 secretion of these clones in response to their stimulatory peptides was detectable (data not shown). All TCC tested were restricted to HLA-DR, as shown by the reduction in NP-specific proliferation when anti-DR antibody was added to the cultures (Fig. 2A).

MHC-II typing of the TCC donor. MHC-II alleles of the TCC donor were determined to be DRB1*0101 and -0301, DRB3*0301, DQA1*0101 and -0101, and DQB1*02 and -0501.

Mapping of T-cell epitopes in the NP of Lassa virus, strain JOS, using TCC. The 60 overlapping peptides that had been

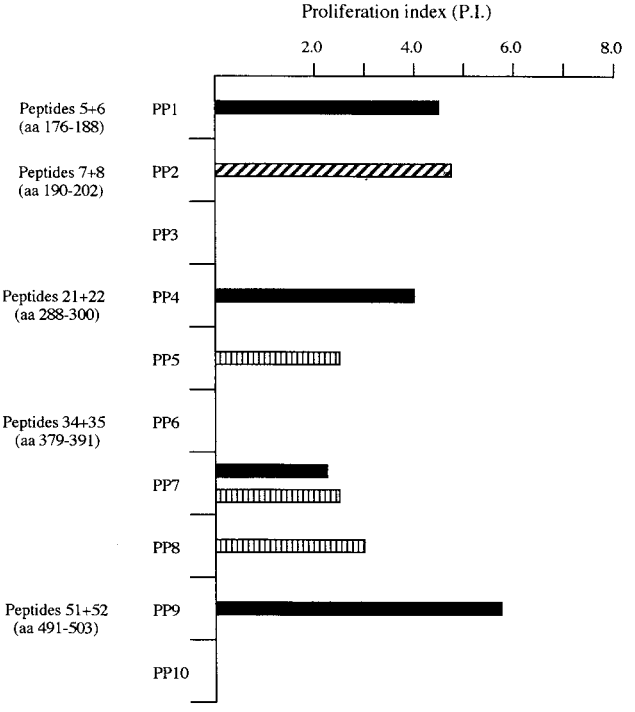

FIG. 1. Proliferative response of PBMC to pooled stimulatory peptides. Assays were run in duplicate (two wells). Each peptide pool (PP) contains six overlapping peptides consisting of 20 aa. PP1 to PP10 span the whole recNP of Lassa virus JOS. Specific proliferation was assessed by calculating [^{3}H]thymidine uptake after a 3-day culture and is expressed as PI (see Materials and Methods). Only statistically significant proliferations are shown (see Materials and Methods). Epitopes of TCC of the TCC donor are located in the overlap of the respective adjacent peptides (Fig. 3), which are contained in the respective peptide pools. ■, donor; ▨, subject 10.2.22; ▥, subject 8.1.10.

TABLE 2. Screening of Lassa NP-specific TCC for reactivity to overlapping peptides spanning aa 141 to 569 of the NP[a]

Peptide	Peptide sequence	PI[b] for:				
		TCC 142	TCC 161	TCC 141/TCC 148	TCC 135	TCC 160
None		1.0	1.0	1.0	1.0	1.0
recNP		193.4	13.3	13.6/3.7	217.8	13.9
P5	AELLNNQ**FGTMPSLTLACLT**	450.6				
P6	**FGTMPSLTLACL**TKQGQVDL	80.3				
P7	TLACLTK**QGQVDLNDAVQAL**		32.2			
P8	**QGQVDLNDAVQAL**TDLGLIY		33.9			
P21	SILKVKK**ALGMFISDTPGER**			32.8/31.9		
P22	**ALGMFISDTPGER**NPYENIL			25.6/24.8		
P34	TLKDAML**QLDPNAKTWMDIE**				51.2	
P35	**QLDPNAKTWMDIE**GRPEDPV				104.5	
P51	SRKYENA**VWDQYKDLCHMHT**					7.1
P52	**VWDQYKDLCHMHT**GVVVEKK					10.4

[a] 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 in boldface.
[b] Values are representative of one of three consistent experiments.

synthesized were first tested for proliferation induction on TCC in 10 pools of 6 peptides each. In a second step, peptides from proliferation-inducing pools were tested individually. Five epitopes were found, which in all cases were contained in two adjacent peptides. They comprise aa 176 to 188, 190 to 202, 288 to 300, 379 to 391, and 498 to 510 of Lassa virus (JOS) (Table 2 and Fig. 1). Two clones, TCC 141 and TCC 148, were found to react to the same two overlapping stimulatory peptides (P21 and P22; Table 2). However, they probably do not recognize the same epitope, because they reacted differently when amino acids in their stimulatory peptides were mutated (see below).

Reactivities of TCC to homologous epitopes of the Lassa virus strain NIG and the arenavirus MOP. A comparison of the JOS epitopes with the homologous sequences in the NP of the Lassa virus strain NIG and the presumably apathogenic African arenavirus MOP is given in Fig. 3. Epitopes comprising aa 176 to 188 and 190 to 200 showed the highest conservation, being identical for JOS and NIG and showing 77 and 69% sequence variation, respectively, for MOP. For epitopes comprising aa 288 to 300 and 379 to 391 the stimulatory 20-aa peptides (homologues to JOS peptides 21 and 22 and 34 and 35) comprising the respective amino acid sequences of NIG and MOP were synthesized. The MOP homologue to peptide 35 (only one amino acid exchange in comparison with JOS) was not tested. The MOP and NIG homologues to JOS peptide 21 stimulated TCC 141, albeit to a lesser extent than P21, but failed to stimulate TCC 148 (Fig. 4A). The overlapping NIG and MOP (identical sequence) homologues to JOS peptide 22 could stimulate both clones but could stimulate TCC 148 only at the highest concentration used (Fig. 4A). TCC 135 could not be stimulated by the NIG or the MOP homologues to JOS peptide 34 (Fig. 4B). In mixing experiments, the NIG and MOP peptides were not found to inhibit proliferation induced by the JOS peptides; rather, there was a slight additive proliferative effect at suboptimal concentrations of the peptides (data not shown).

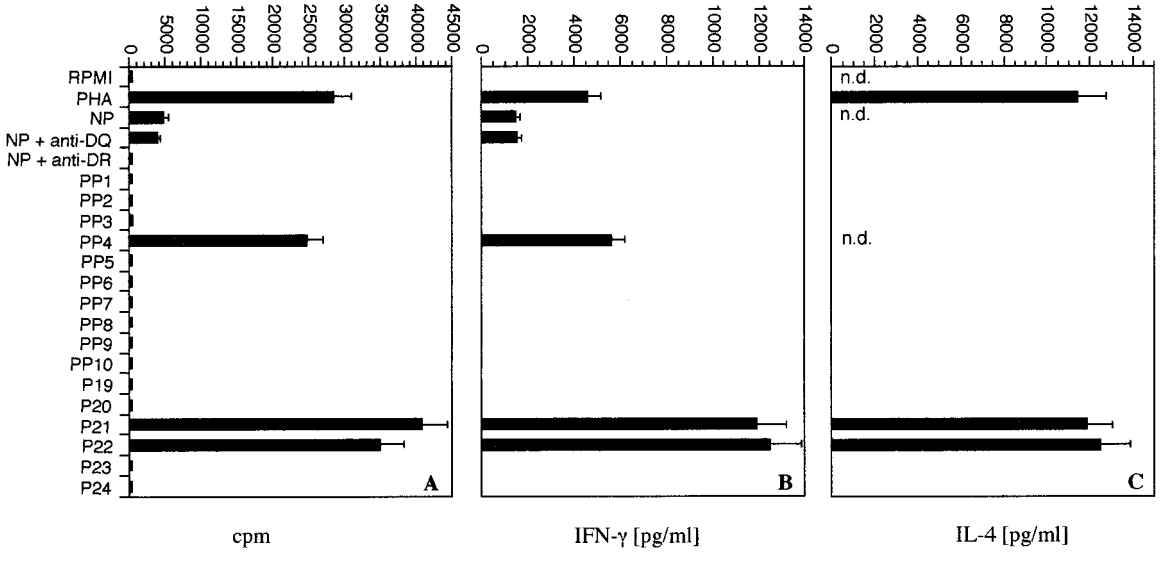

FIG. 2. Proliferation assays of TCC 148 with different stimuli and measurement of cytokines. Assays were run in duplicate (two wells each); the results of one of two consistent experiments are shown. (A) Proliferation ([^{3}H]thymidine counts per minute) in response to PHA, recNP, peptide pools (PP; containing six stimulatory, overlapping peptides each), and individual peptides. Blocking of proliferation was with anti-DR but not anti-DQ MAbs. (B and C) Production of IFN-γ and IL4 in response to specific and nonspecific stimuli. Error bars, two standard deviations.

FIG. 3. Alignment of JOS NP CD4 T-cell epitopes with heterologous arenaviruses. Boxed regions indicate overlap of stimulatory peptides; the numbering of peptides is according to Table 2. Amino acids in boldface differ from those in the JOS NP sequence. Numbers below boxes indicate the positions of the first and last amino acids, as in Lassa virus, strain JOS (EMBL accession no., JO 4324). Sequences of NIG and MOP have EMBL accession no. X52400 and M33879, respectively.

DISCUSSION

Only indirect evidence of the pivotal role that may be played by T-cell immunity in the recovery of humans from acute Lassa virus infection and in protection from reinfection has been accumulated. Lassa fever patients are viremic throughout the acute phase of the disease in the presence of high titers of nonneutralizing antibodies (11). Late in convalescence, i.e., 3 to 4 months after the acute phase, in up to 88% of patients very low titers of N antibodies (9, 28), which preferentially neutralize Lassa virus variants isolated from the same geographical area, appear (19). It is presently not clear to what extent these low-titer N antibodies protect from homologous or heterologous reinfection in areas of endemicity. Presumably asymptomatic reinfections have been shown to occur, as judged by a rise of antibody titers measured by IIF. On the other hand, 6% of IIF-seropositive persons also serorevert annually in areas of endemicity (20), and it has not been investigated if their sera retain a neutralizing activity.

Vaccination of animals, including nonhuman primates, with heterologous arenaviruses (7, 10, 13) or recombinant vaccinia viruses expressing Lassa virus structural proteins (1, 3, 7, 21) has resulted in full or partial protection from disease, respectively. Vaccinia virus constructs expressing the Lassa virus NP protected 94 to 100% of guinea pigs but only 25% of monkeys, whereas constructs expressing the glycoproteins protected all monkeys and 79% of guinea pigs. Infection leading to transient viremia, often associated with mild disease symptoms, could not be prevented by the recombinant vaccines. Prechallenge N antibodies could not be detected in any of the experiments, and the titers of N antibodies postchallenge were reported to be very low, not exceeding 1:2 (7). Cellular immunity conferred by vaccination was investigated in the guinea pig model of Lassa virus infection, where adoptive spleen cell transfer of animals immunized with different arenaviruses protected naive animals from homologous and heterologous infection (10). Interestingly, this protection, which was shown to be conferred by $CD8^+$ cytotoxic cells, was effective only when the cells were transplanted shortly after the initial immunization. Furthermore, highly virulent Lassa virus strains did not induce a CTL response. Recently it was reported that vaccination of mice with a recombinant vaccinia virus expressing the glycoprotein of Lassa virus protected from LCMV infection in the absence of N antibodies (16). Cytotoxic $CD4^+$ TCC were shown to mediate this protection, and it was speculated that this type of cells could generally be involved in cross-protective immunity against heterologous arenavirus infections. Taken together, these experiments imply that T-cell immunity plays a major role in the resolution of acute Lassa virus infection and an important role in the prevention of reinfection.

Because of logistic problems involved with working in the remote areas where Lassa fever is endemic, data on human T-cell responses to Lassa virus infection have so far not been generated. For the first time we show here that Lassa virus antibody-seropositive and also -seroreverted persons from an area of endemicity have very strong $CD4^+$ T-cell responses against the recNP of Lassa virus, strain JOS. Furthermore, in some individuals the PBMC could be stimulated directly with peptides comprising the CD4 T-cell epitopes mapped for one MHC-II typed donor. The study subjects had been identified 6 years earlier on the basis of high Lassa virus antibody titers ranging from 1:160 to 1:2,560; however, all were free of Lassa fever symptoms at that time. Monitoring our study subjects over a period of 6 years revealed for all except one a decline in antibody titer, with two individuals having seroreverted. We therefore conclude that the observed virus-specific lymphocyte proliferation reflects the expansion of memory $CD4^+$ cells which were generated at least 6 years ago and which had not been boosted since. These observations indicate that $CD4^+$ cells play an important role in controlling this human arenaviral infection and are in line with a large body of data generated from animal experiments with the prototype arenavirus, LCMV. N antibodies are absent in acute LCMV infection, possibly because LCMV-infected B cells are killed by virus-specific CTLs (25). Acute LCMV infection (strain Armstrong; C57BL/6 mice) induces strong $CD8^+$ and $CD4^+$ T-cell responses in mice, with 1/35 to 1/160 of all $CD4^+$ T cells being virus-specific Th1 cells and 1/400 being Th2 cells (32). Other studies have found most of the LCMV-induced $CD4^+$ cells to be of the Th1 type, with 1/47 of all $CD4^+$ T cells being specific for an LCMV glycoprotein epitope and 1/124 being specific for an NP epitope (12, 23). Although LCMV infection induces a very strong $CD8^+$ T-cell response, with 50 to 70% of all $CD8^+$ cells in the spleen of the mouse being virus specific at the peak of infection (2, 22), without CD4 help the expansion of CTLs is insufficient to control fast-replicating LCMV strains (18). Furthermore, $CD4^+$ cells are required for the maintenance of the $CD8^+$ T-cell memory (23). In the absence of $CD4^+$ cells chronic LCMV infection with clone 13 leads to the persistence of virus-specific $CD8^+$ cells without measurable effector function (33). Furthermore, CD4-deficient mice have reduced levels of memory CTLs after immunization against LCMV and

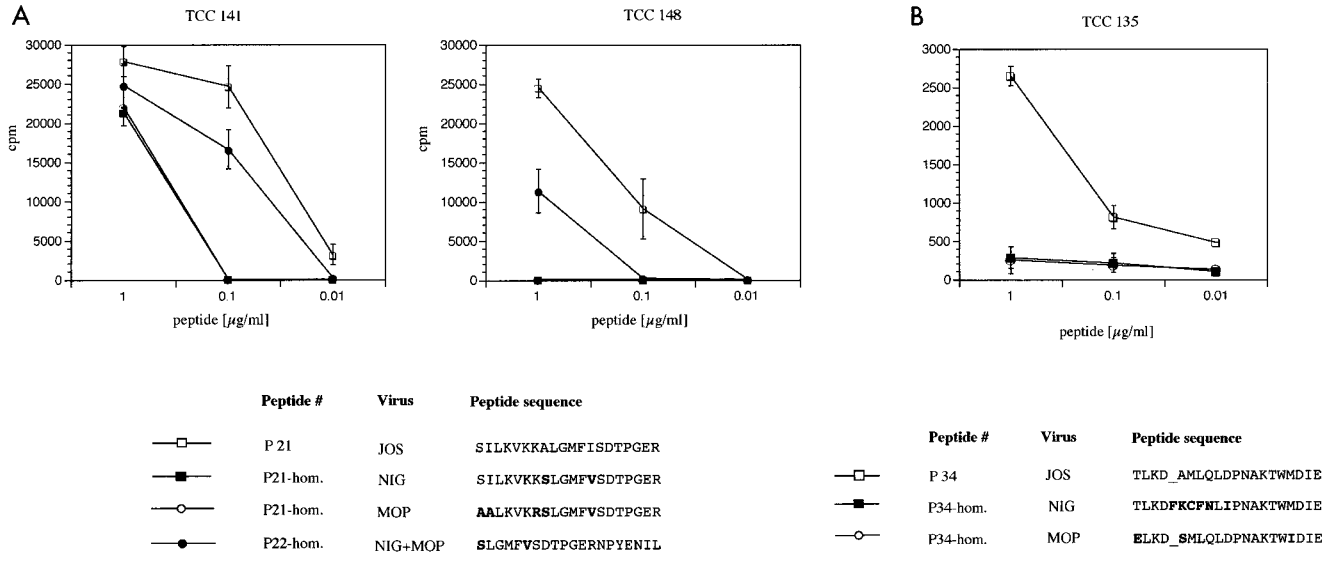

FIG. 4. Reactivity of TCC 141 and 148 to stimulatory peptides from JOS and homologous peptides derived from the sequences of NIG and MOP. Assays were run in triplicate; the representative results of one of three independent experiments are shown. Error bars, two standard deviations. Reactivity of TCC 135 to stimulatory peptides from JOS and homologous peptides derived from the sequences of NIG and MOP. Assays were run in duplicate; the representative results of one of two independent experiments are shown. Error bars, two standard deviations. Amino acids in bold type differ form the Josiah sequence.

show diminished resistance to subsequent viral challenge (30). After viral clearance in mice, the frequency of $CD4^+$ memory cells specific for a single LCMV NP epitope was measured as 1/1,126 by enzyme-linked immunospot assay for IFN-γ (32), or as 1/1,200 for virus-specific $CD4^+$ cells (29). In our study, the PBMC of the TCC donor, who had not been reinfected with Lassa virus for at least 6 years, showed a specific proliferation to pools of stimulatory peptides, each containing one of the five TCC epitopes which had been identified. Because 10^5 PBMC were seeded for the proliferation assays, comprising approximately 3×10^4 $CD4^+$ cells, the frequency of $CD4^+$ memory cells for individual peptides is on the order of >1/30,000; for the whole NP it is >1/6,000. These values are comparable to those from the murine models. Obviously, Lassa virus and LCMV induce similar, predominantly T-cell-dependent immune responses, and our data suggest that the importance of $CD4^+$ cells in Lassa virus infection might be as great as that described for LCMV.

Arenaviruses tend to show considerable variation of their nucleotide and amino acid sequences in nature. To date the structural genes of only two Lassa virus strains have been completely sequenced. The amino acids of the structural proteins of strain JOS from Sierra Leone and of strain NIG from Nigeria were shown to differ by approximately 15%. A comparison of PCR fragments generated from a number of isolates from Sierra Leone and Liberia showed amino acid variations of up to 6.3% in the glycoprotein (J. C. S. Clegg, S. M. Wilson, and G. Lloyd, Abstr. 8th Int. Conf. Negative Strand Viruses, abstr. 143, p. 111), and we have recently amplified a viral sequence from a Lassa fever patient in the Republic of Guinea which varied from both the JOS and NIG strains by more than 10% in the amino acid sequence of the NP (27). Investigation of the cross-protective potential of T-cell responses against different Lassa virus strains is therefore important for understanding the natural history of the disease and for evaluating strategies to design recombinant vaccines. To this end, we investigated the cross-reactivity of our TCC with the homologous epitopes from the Lassa strain NIG and the presumably apathogenic east African arenavirus MOP. We were able to perform these experiments with three TCC, of which two were stimulated by the same overlapping JOS peptides (TCC 141 and 148). Further experiments indicate that these clones probably do not recognize the same epitope, because one clone (TCC 141) showed reduced proliferation when stimulated with the homologous peptides of NIG and MOP, whereas the other (TCC 148) did not react. The reactivity of this clone could be partly restored with an overlapping peptide (Fig. 4A; P22-hom). The latter finding could be due to the fact that the amino acids contributing to the contact between the P22 homologue and the MHC-II molecule are different from those contributing to the contact between the P21 homologue and the MHC-II molecule rather than due to a second T-cell epitope being offered to the T-cell receptor. The third clone (TCC 135) also showed a nearly abolished reactivity with the homologous NIG and MOP peptides, with only one amino acid exchange in the putative epitope for the P34 homologue of MOP (Fig. 4B; Fig. 3). Findings similar to ours have been reported for other viruses. In Dengue virus infection, cytotoxic $CD4^+$ TCC were cross-reactive against three Dengue serotypes, because of perfect conservation of their epitopes. However, two amino acid substitutions in the homologous epitope of the fourth serotype abolished the reactivity of the clones (14). In chronic hepatitis C virus infection, the reactivity of $CD4^+$ TCC was greatly reduced after single-amino-acid point mutations in the epitopes of nonstructural protein NS3, which were observed to occur as possible T-cell escape variants over a period of time (31). The degree of conservation of immunodominant T-cell epitopes and/or MHC binding regions between different virus strains might be pivotal for cross-protective immunity in a predominantly T-cell-controlled infection such as Lassa fever. It will therefore be important to assay $CD4^+$ T-cell epitopes on the NP and glycoproteins of Lassa virus presented by common MHC-II molecules. Furthermore, their degree of variation in naturally occurring Lassa virus variants should influence the design of recombinant vaccines against Lassa fever.

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