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Synthetic peptides based on *Chlamydia trachomatis* antigens identify cytotoxic T lymphocyte responses in subjects from a trachoma-endemic population

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SUMMARY

$CD8^+$ cytotoxic T lymphocytes (CTL) recognize peptide antigens in the context of class I MHC antigen molecules. To identify peptides capable of eliciting anti-*Chlamydia trachomatis* CTL responses, 13 synthetic peptides conforming to human leucocyte antigen (HLA)-B8- or -B35-predicted binding motifs were synthesized using sequences based on *C. trachomatis* major outer membrane protein (MOMP) and heat shock protein 60 (hsp60). Two of 11 HLA-B35-predicted binding peptides were able to stabilize HLA-B35 in an *in vitro* binding assay. All peptides were tested in CTL assays using peripheral blood mononuclear cells (PBMC) isolated from 26 HLA-B8 or -B35 individuals resident in a trachoma-endemic community. Responses to MOMP and hsp60 peptides were identified in a minority of both HLA-B8 and -B35 individuals. Two of 12 HLA-B8 subjects responded to MOMP and 1/13 to hsp60 peptides. Responses in HLA-B35 subjects were similar, 1/13 subjects responding to MOMP and 2/13 to hsp60 peptides. CTL responses were observed only in children resolving current infection and in adults without scarring of the conjunctiva. These results suggest that anti-chlamydial CTL occur at low levels in peripheral blood, but may be important in the resolution of naturally acquired human ocular chlamydial infection.

Keywords cytotoxic T lymphocyte trachoma HLA

INTRODUCTION

Chlamydia trachomatis is an obligate intracellular bacterium responsible for the most common form of infectious blindness, trachoma [1]. When sexually transmitted it is also a major cause of salpingitis and infertility [2]. Whilst antibiotic treatment of individual cases is effective in both ocular and genital infection, such treatment of 'at risk' communities is not possible. An effective vaccine is therefore desirable and would have significant public health benefits.

Protection in murine models by immune T cells has highlighted the effectiveness of cellular immunity against this pathogen [3–5]. Studies to delineate the contribution of $CD4^+$ and $CD8^+$ T cells in resolution of murine infection suggest that cells of either subset can mediate resolution and that $CD4^+$ T cells have a quantitatively greater role [6–8]. Using β2m, MHC class II or CD4 gene knockout mice, Morrison *et al.* [9] demonstrated an absolute requirement for MHC class II-restricted responses in protection from genital chlamydial infection. In contrast, MHC class I-restricted responses were not essential. However, use of gene knockout mice in such models of infection does not preclude a role for class I-restricted $CD8^+$ T cells in the immunocompetent host or in immunity to naturally acquired infection. Furthermore, murine anti-chlamydial MHC class I-restricted cytotoxic T lymphocytes (CTL) that lyse infected cells *in vitro* and protect against *in vivo* challenge have been identified [10,11]. In a wider context, studies on other intracellular bacteria have demonstrated the importance of CTL in clearance of infection and in prolonged survival following challenge [12–15]. Therefore, as a prerequisite to the development of an anti-chlamydial vaccine which induces a CTL response in humans, it is important to identify whether CTL responses occur following exposure to natural infection.

Genetic and immune factors were studied in a trachoma-endemic population and focused on the polymorphic HLA class I and II regions [16,17]. Scarring trachoma was associated with the class I allele HLA-A*6802. All other HLA class I and II types tested were equally distributed between individuals with severe conjunctival scarring and controls. The findings suggest that HLA class I-restricted CTL involved in protection are not restricted to a few or limited number of HLA antigens/epitopes, and that

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HLA-A*6802-restricted effector cells may play a role in the pathogenesis of trachoma. Two other HLA class I alleles associated with immunopathogenic sequelae of genital chlamydial infection have been identified [18,19]. However, immunopathogenic *Chlamydia*-specific effector cells restricted by the HLA alleles identified in these studies have yet to be identified. Therefore we sought evidence of class I-restricted CTL which were restricted by HLA alleles not associated with pathological trachomatous responses. $CD8^+$ CTL responses to three HLA-B8- and 11 HLA-B35-restricted synthetic peptides based on sequences derived from *C. trachomatis* serovar A major outer membrane protein (MOMP) and heat shock protein 60 (hsp60) were tested in 26 individuals from a trachoma-endemic population.

PATIENTS AND METHODS

Patients

Subjects were selected on the basis of HLA-B antigen type from a trachoma-endemic population in The Gambia [16]. The HLA types -B8 and -B35 were found in $\approx$ 20% and 30% of individuals, respectively, and one or the other would be present in almost 50% of individuals [16,20]. Twenty-six HLA-B8 or -B35 subjects were selected in total, 12 adults with severe scarring of the conjunctiva and 10 adults without evidence of trachomatous scarring. The remaining individuals were children identified as likely to resolve ocular infection, i.e. with clinical signs of active trachoma but without evidence of chlamydial antigen in ocular swabs. Longitudinal studies of the natural history of trachoma have shown that clinical signs persist for some weeks after the clearance of ocular chlamydial infection and that the clinical signs of trachoma resolve rapidly once subjects become negative for chlamydial antigens [21,22].

Synthetic peptides and MHC binding

All peptides were synthesized by solid-phase synthesis (Applied Biosystems Inc, Warrington, UK). Thirteen peptides, of eight or nine amino acids in length, corresponding to *C. trachomatis* antigens MOMP (serovar A) and hsp60 were synthesized. The peptides covered sequences which matched motifs predicted to bind HLA-B8 or -B35 from these proteins (Table 1). HLA-B8-predicted binding peptides conformed to K/R at positions 3 and 5 and L/I at positions 8 or 9. HLA-B35-predicted binding peptides based on *C. trachomatis* sequences conformed to the motif described by Hill *et al.* [23]. Only sequences with P at position 2 and I, L, M**,** or Y at positions 8 or 9 were synthesized. *Chlamydia trachomatis* serovar A-based peptides were kindly provided by Dr P. Bowness (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK). Purity was determined by reverse phase high performance liquid chromatography (HPLC; Gilson HPLC analyser; Anachem Ltd, Luton, UK). Purity ranged from 58% to 99%, with the majority (9/13) > 80% pure. The 11 HLA-B35-predicted binding peptides were tested at 5 μM for binding to HLA-B35 in a T2-HLA-B35 assembly assay [24]. Both binding and non-binding peptides were included in CTL assays. In addition, previously described CTL epitopes of influenza virus-nucleoprotein (NP) and -matrix (M) protein, which were restricted by HLA-B8 and -B35, were synthesized and used as positive controls for CTL stimulation and target cell lysis [25,26]. Influenza peptides used were: HLA-B8-restricted NP peptide ^{380}EL**R**S**R**YWA**I**^{388} described by Sutton *et al.* [26] and was synthesized commercially (Calbiochem-Novabiochem (UK) Ltd, Nottingham, UK), and for HLA-B35 the matrix peptide ^{127}NA**S**CMGLI**Y**C^{136}, described by Dong *et al.* [25], a kind gift of Dr M. Plebanski (Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK).

Table 1. Sequences of peptides synthesized based on *Chlamydia trachomatis* antigens corresponding to the HLA-B8 and -B35 binding motifs

HLA type	Peptide	Sequence	Antigen	Amino acid position	*In vitro* binding
HLA-B8	1	QS**K**P**K**VEE**L**	MOMP A	198–207	ND
	7	GP**K**G**R**HVV**I**	hsp60	32–40	ND
	8	LG**K**A**K**KV**I**	hsp60	317–324	ND
HLA-B35	2	N**P**AEPSLM**I**	MOMP A	5–13	+
	3	E**P**SLMIDG**I**	MOMP A	8–16	–
	4	N**P**AYGKH**M**	MOMP A	86–93	–
	5	Q**P**KLAKPV**L**	MOMP A	288–296	–
	6	K**P**VLDTTT**L**	MOMP A	293–301	–
	7	G**P**KGRHVV**I**	hsp60	32–40	–
	9	N**P**MDLKRG**I**	hsp60	113–121	–
	10	K**P**VQHHKE**I**	hsp60	136–144	–
	11	N**P**ETQECV**L**	hsp60	207–215	–
	12	L**P**GGGTAL**I**	hsp60	412–420	+
	13	I**P**TLEAF**L**	hsp60	423–430	–

MOMP A, Serovar A major outer membrane protein; hsp60, serovar A heat shock protein 60; residues in bold indicate anchor residues for the HLA-B8 and -B35 binding motifs. Peptide 7 has residues which correspond to both the HLA-B8 and -B35 motifs. ND, Not done. +, Stabilization of HLA assembly by peptide *in vitro*; –, peptide unable to stabilize HLA assembly *in vitro*. For CTL assays peptides were pooled as follows: HLA-B8 MOMP A, P1 only; hsp60, P7, 8; HLA-B35 MOMP A, P2, 3, 4, 5, 6; hsp60, P7, 9, 10, 11, 12, 13.

Effector cells

Peripheral blood mononuclear cells (PBMC) were isolated from 20 ml venous blood samples by centrifugation over Lymphoprep (Nycomed, Birmingham, UK) and were typed for HLA class I antigens by standard microlymphocytotoxicity tests using a panel of 120 defined anti-sera [27]. PBMC for CTL assay were incubated for 1 h with 10–100 μM of each peptide or a pool of peptides as indicated (Table 1). Due to the number of cells available for stimulation, peptides were pooled according to *C. trachomatis* antigen and HLA binding motif (Table 1). The range of peptide concentrations selected was based on the observations of Aidoo *et al.* [28], who found CTL responses to peptides within 10–100 μM. Peptides which were used at < 100 μM were maximal concentrations and were dependent on the concentration of peptide after synthesis. Peptides were tested at the following final concentrations: 100 μM, P1, 2, 4, 5, 8, 11, 13; 50 μM, P12; 40 μM, P3, 7; 20 μM, P6, 25; 10 μM, P12. PBMC were then cultured without washing in 24-well plates at 2×10^6 cells/ml in 1 ml RPMI 1640 supplemented with 10% autologous plasma, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 25 ng/ml recombinant human IL-7 (rhIL-7; R & D Systems, Abingdon, UK) [29]. Seventy-two hours later rhIL-2 (Hoffman La-Roche, Basel, Switzerland) was added to a final concentration of 5 U/ml. Fresh autologous growth medium containing 5 U/ml rhIL-2 was added to the cultures every 3–4 days.

Cytotoxic T cell assay

Cytotoxicity assays were performed in triplicate cultures at two effector-to-target cell ratios (E:T) of 40:1 and 20:1 [28]. The number of E:T ratios tested was limited by the number of cells available following isolation and stimulation. Assays for CTL activity were routinely performed after 7 and 14 days of culture. CTL responses at 20:1 were concordant with those at 40:1. Target cells were either autologous or HLA-B antigen-matched Epstein–Barr virus-transformed B cell lines (B-LCL) which were ^{51}Cr-labelled. Targets were pulsed with individual or pools of peptides identical to those used to stimulate the effector cell population for 1 h before excess peptide was removed by washing in serum-free RPMI 1640. After 4 h incubation with effector cells ^{51}Cr release was determined by collection of 20 μl of cell-free supernatant onto glassfibre filter mats followed by liquid scintillation counting (1205 Beta plate; Pharmacia Biotechnology, Milton Keynes, UK). The percentage specific lysis was calculated as follows: percent specific lysis $= 100 \times$ ((test ct/min $-$ spontaneous ct/min)/(maximum ct/min – spontaneous ct/min)).

Statistical analysis

A paired *t*-test was used to test for differences between lysis of unpulsed targets and peptide-pulsed targets by effector cells, i.e. to test for the effect of the addition of peptides. For statistical analysis a constant was added to each of the values, to correct for negative values, and the data log transformed. Each data set was then tested for continuous distribution before application of Students *t*-test. All data were continuously distributed.

Ethical approval

This study was approved by the Joint Scientific and Ethical Committee of the MRC Laboratories, Fajara, and the Government of the Gambia. Informed consent was obtained from all study participants or their parents/guardians.

RESULTS

HLA-B35 binding assay

Two HLA-B35-restricted peptides which stabilized HLA class I assembly *in vitro* (Table 1) were identified, one peptide from each of the antigens selected (MOMP and hsp60).

CTL response to predicted HLA-binding peptides

Stimulation by HLA-B35-restricted peptides resulted in significant increases of the difference between CTL lysis of peptide-pulsed targets in response to MOMP ($P = 0{\cdot}018$) and hsp60 ($P = 0{\cdot}038$) and unpulsed targets. Peptides corresponding to the HLA-B8 motif from both MOMP A (P1) and hsp60 (P7 and P8) did not significantly increase the mean difference in

Table 2. Mean specific lysis of HLA-B8 or -B35 peptide-pulsed targets by peripheral blood mononuclear cells (PBMC) stimulated with peptides. CTL activity assayed on day 14 and at an effector:target ratio of 40:1

HLA type	Stimulating peptides	Targets	Mean % specific lysis	95% CI	*n*	*P*
HLA-B8	MOMP A	Pulsed	7·9	–2–19	12	0·12
		Unpulsed	3·9	–1·4–9·4		
	hsp60	Pulsed	5·4	–3–13·2	13	0·2
		Unpulsed	3	–4·4–10·9		
HLA-B35	MOMP A	Pulsed	12·3	6–19	12	0·018
		Unpulsed	8·7	2·2–15·6		
	hsp60	Pulsed	12·4	5–20·5	13	0·038
		Unpulsed	8·9	0·6–17·8		

P value (one-sided) obtained from paired Student's *t*-test (lysis of pulsed targets – lysis of unpulsed targets); MOMP A, serovar A major outer membrane protein peptides; hsp60, serovar A heat shock protein 60 peptides; *n*, number of sample pairs; 95% CI, 95% confidence interval of the mean.

Table 3. Number of individuals from each clinical group with evidence of CTL (CTL activity assayed on day 14 and effector:target of 40:1)

		Clinical group			
HLA type	Peptides	S	C	R	Total
HLA-B8	MOMP A	0/4	1/6	1/2	2/12
	hsp60	0/4	1/7	0/2	1/13
HLA-B35	MOMP A	0/8	0/3	1/2	1/13
	hsp60	0/8	1/3	1/2	2/13

S, Adults with trachomatous scarring; C, adult endemic controls without evidence of trachomatous scarring; R, children with inflammatory trachoma, with some evidence of resolving infection. MOMP A, Serovar A major outer membrane protein peptides; hsp60, serovar A heat shock protein 60.

specific lysis of peptide-pulsed targets compared with unpulsed targets (Table 2).

Peptide-specific CTL responses in which differences between pulsed and unpulsed targets were >10% specific lysis were considered to provide evidence of peptide-specific CTL [28]. The subjects were then divided into their respective clinical categories, and in a number of individuals *C. trachomatis* peptide-specific CTL responses with a difference of >10% specific lysis were identified. Two of 12 HLA-B8 subjects responded to MOMP and 1/13 to hsp60 peptides. Similar CTL responses in HLA-B35 subjects were observed; 1/13 subjects responded to MOMP and 2/13 to hsp60 peptides (Table 3). The differences in specific lysis by CTL from these six subjects are represented in Fig. 1. The remaining subjects had differences <10% specific lysis. By dividing subjects on the basis of clinical status, CTL responses were found only in adult controls and in children resolving current infection.

CTL were elicited in response to the HLA-B8-restricted influenza-NP and the HLA-B35 influenza-M peptide. CTL responses to the HLA-B8- and -B35-restricted influenza peptides were identified in 77% (10/13) of HLA-B8 individuals and 15% (2/13) of HLA-B35 individuals.

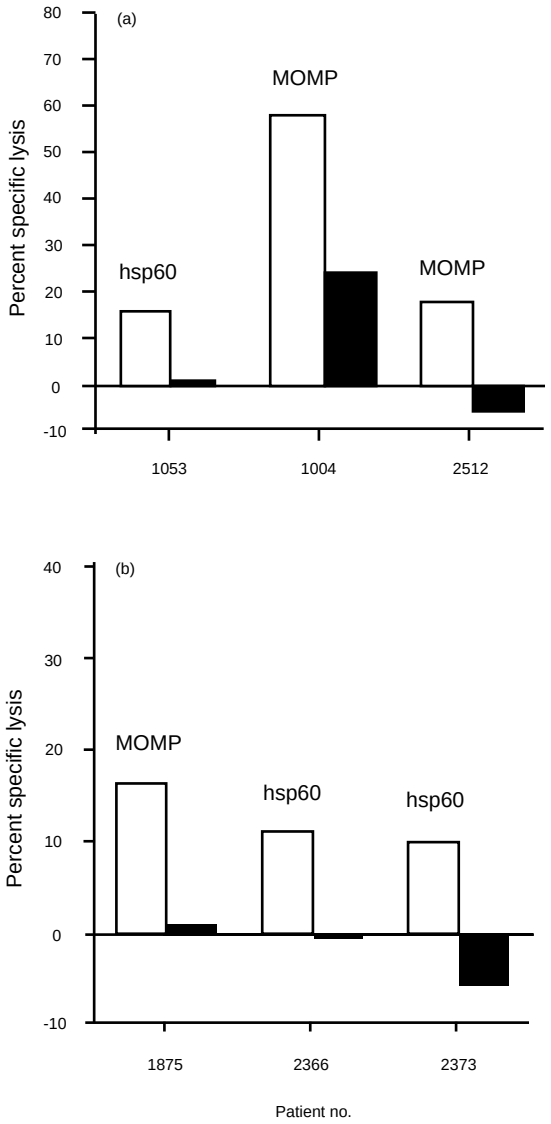

Fig. 1. HLA-B8- (a) and -B35-restricted (b) *Chlamydia trachomatis* peptide cytotoxic T lymphocyte (CTL) responses. Results of CTL assays performed at day 14 with differences of >10% specific lysis between peptide-labelled targets and targets without peptide at an E:T of 40:1. Autologous or HLA-B-matched Epstein–Barr virus-transformed B cell lines (B-LCL) served as target cells. Patients 2366, 2373 and 2512 were subjects with evidence of resolving ocular infection. Patients 1053, 1004 and 1875 were subjects from a trachoma-endemic area without evidence of current infection or scarring of the conjunctiva. □, Pulsed; ■, unpulsed. MOMP, Major outer membrane protein; hsp60, serovar A heat shock protein 60.

DISCUSSION

These data identify human CTL responses to epitopes in antigens of *C. trachomatis*. CTL responses were elicited only in children resolving current infection and in adults without evidence of conjunctival scarring. The detection of *Chlamydia* peptide-specific CTL activity in a minority of individuals may have a number of explanations. Specific CTL may be present at very low frequency in the peripheral circulation, and require further expansion *in vitro* before CTL activity can be readily detected. This could be circumvented by longer periods of *in vitro* stimulation.

The selection of study subjects is vitally important, as CTL may only be found transiently in the peripheral circulation following 'boosting' either by infection or by exposure to *C. trachomatis*. In addition, the *in vitro* culture of a mixed cell population could inhibit the expansion of the desired *Chlamydia*-specific CTL. These problems can be minimized by selection of the appropriate study subjects and cloning/limiting dilution analysis of the CTL.

Previous attempts to demonstrate *Chlamydia*-specific CTL in humans have failed to detect either significant specific activity [30] or specificity [31–33]. Using *C. trachomatis*-infected HLA-matched primary fibroblasts as both antigen- presenting cells and target cells, specific lysis was identified in 1/8 subjects. However, this was complicated by a high background of non-specific lysis, therefore we utilized an alternative MHC peptide binding motif approach. CTL were demonstrated in 6/26 subjects in the absence of high non-specific background lysis. The use of peptide antigens precludes the need for antigen processing, and CTL should only be elicited from PBMC if the peptide presented forms an epitope to which that individual has specific immunological memory. *In vitro* studies

in murine cells demonstrated that productively and persistently *Chlamydia* spp.-infected cells can present chlamydial epitopes and act as targets for murine CTL [10,11,34]. Future studies should investigate whether peptide-specific CTL lyse *Chlamydia*-infected cells.

CTL responses to the hsp60 pool of peptides raises a number of interesting questions. Homologues of hsp60 are highly conserved between species, and it has been proposed that bacterial hsp60 peptides are the stimulus/target for immunopathological responses [35]. Alternatively, they can also be the target for protective responses [35,36]. The chlamydial based hsp60 peptides synthesized contain at least one amino acid substitution compared with the human homologue. In 3/8 peptides this leads to an amino acid change in an MHC binding anchor residue. Four of five remaining peptides have at least four or more substitutions which affect interaction with the T cell receptor. As no hsp60 peptide responses were observed in individuals with scarring sequelae, we assume that the CTL responses observed were directed against chlamydial and not self hsp60 peptides.

In conclusion, two HLA-B35 binding peptides were identified. We screened PBMC from 26 individuals for CTL responses with all peptides conforming to the HLA-B8 or -B35 motifs. Preliminary results indicate that peptide-specific CTL can be identified in 6/26 individuals. Peptide-specific CTL were found only in those subjects free from conjunctival scarring and in children resolving infection. Further studies of CTL responses from a larger number of subjects exposed to *C. trachomatis* may now be undertaken.

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