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Identification of T helper cell-recognized epitopes in the chitinase of the filarial nematode *Onchocerca volvulus*

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Abstract

T helper cell-recognized epitopes were determined in chitinase of *Onchocerca volvulus*, a vaccine candidate protein. The proliferation of splenic T cells of mice immunized with recombinant protein was tested with a library of chitinase-peptides of 16 amino acids with termini overlapping by 12 amino acids, and a library of "designer peptides", i.e. sequences identified with three epitope-predicting algorithms. Fourteen epitope-bearing stretches were identified with the peptides of the overlapping library. Testing of the designer peptides partially confirmed these data and revealed additional epitopes. Five clusters of epitopes were identified for the creation of peptide or minigene DNA vaccines with good potency and potential range of MHC allele presentation.

Keywords: Peptide vaccine; Chitinase; MHC Class II-recognized epitopes

1. Introduction

River blindness, or onchocerciasis, is a major health problem in several tropical countries, mostly in Africa, south of the Sahara. More than 18 million persons are infected with the filarial nematode *Onchocerca volvulus*, the causative agent of this debilitating disease [1]. The nematode develops in subcutaneous nodules within the skin and produces large numbers of microfilariae, i.e. larvae which migrate in the skin and eyes of the patients. Inflammations induced by the microfilariae lead to skin diseases and to eye lesions, which may result in blindness. Programs for the control of the *Simulium* vector and ivermectin therapy have well suppressed the parasite in many formerly endemic areas [2], and reduced the prevalence and intensity of infections in many other regions. However, river blindness outbreaks might recur unless vector-controlling measures and chemotherapy campaigns are maintained continuously. Since these efforts require relatively costly and sophisticated logistics, an effective vaccine against *O. volvulus* is highly desired, to complement available tools for onchocerciasis control on the long run.

Vaccinations with several protein antigens of filarial nematodes induced significant partial protection in animal models of filariasis [3–7]. These experimental vaccines probably target early larval stages during the process of invasion, growth and moulting, before the worms reach sexual maturity after several months. A good target for vaccine development is the chitinase of the third larval stage, an antigen which is synthesized and secreted during the early phase of infection and during the first molt [8,9]. Filarial chitinases degrade chitinous structures of the nematode's eggshell and possibly of the cuticle, and therefore have a key function in development of the worms. Filarial chitinases belong to the Class 18 hydrolase family with a TIM-barrel structure and have great structural similarities to the chitinase of the pathogenic fungus *Coccidioides immitis* [10], with the exception that the *O. volvulus*-chitinase possesses a chitin-binding domain. Antibody responses to certain filarial proteins, which were later identified as chitinases, have been associated with protective immunity both in humans [11–13] and in an animal model [14]. Wang et al. [7] and Harrison et al. [15] found that recombinant chitinase of the filaria *Brugia malayi* or a DNA vaccine for the chitinase of *O. volvulus*, respectively, induced partial

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protection against challenge infections in rodents. However, all of the reported studies of those experimental vaccines against filarial nematodes yielded only partial protection. Further vaccines are being pursued to enhance immunoprotective mechanisms involving antibodies and helper T cells which regulate the magnitude of the effector responses and provide memory. Cellular immunity plays an important role in protective immunity against filarial infections as shown in mouse models, where depletion of $CD4^+$ cells leads to an increase of worm burdens [16].

In order to develop more effective vaccines against filariasis, we have undertaken to discover helper T cell-recognized epitopes in the chitinase of *O. volvulus*. There are many ways in which vaccines utilizing such epitopes might come to be exploited. An expanded helper T cell response against a single epitope can potentiate both antibody responses and the licensing of dendritic cells which in turn amplify the cytotoxic T cell response [17,18]. Immunological memory is also a function of the helper T cell population. One can envision immunizations first with one helper T cell-recognized epitope, followed with secondary immunizations with either recombinant proteins or DNA vaccines. Expansion of the helper T cell response to even a single T helper cell epitope, can potentiate the secondary response to a wide range of antibody- or cytotoxic T cell-recognized epitopes [19].

In order to identify the best T helper cell-recognized epitopes in the chitinase of *O. volvulus* for the development of peptide vaccines, we have undertaken a systematic study of peptides from two groups. The first group was a library of overlapping peptides through the entire sequence of the antigen, and the second consisted of designer peptides synthesized to include putative MHC Class II-presented motifs. The peptides in the overlapping library were each 16 amino acids (aa) in length, with 12 aa overlaps between successive peptides in the sequence. Sets of designer peptides were prepared according to three algorithms based on consensus motifs for murine MHC Class II epitopes. A priori, one might suggest that such designer peptides, being tailored for the motif sitting within the antigenic peptide-binding site, might have the greatest potency. Such peptides with clustered, overlapping epitopes might be presented by a wider range of MHC Class II alleles and thus lead to the most clinically useful vaccine. On the other hand, if individual potent peptides are presented by respectively different human MHC Class II alleles, then a basket of such peptides might be needed for a clinically useful vaccine.

2. Materials and methods

2.1. Cloning and expression of O. volvulus-chitinase

The cDNA of *O. volvulus*-chitinase (EMBL accession no. L42021; provided by Prof. Dr. A.E. Bianco, Liverpool School of Tropical Medicine) was amplified by PCR with primers (sense: CCGGATCCTATGTTCGAGGATGCTATTATAC; antisense: TTTAAGCTTTTCATCGCATTCACCAAA) containing unique *Bam*HI and *Hin*dIII restriction sites. The reaction was designed to yield the a cDNA lacking the codons for the first 15 amino acids of the signal sequence. The amplification products were cloned in a T-overhang vector (pGEM-T, Promega, Mannheim, Germany) and subcloned for expression into the plasmid pQE30 (Quiagen, Hilden, Germany), using the *Bam*HI and *Hin*dIII sites. This plasmid allows expression of polypeptides with a 6His-tag suitable for affinity purification of the expression product. The plasmids were transformed into the *Escherichia coli* strain XL-1 Blue and recombinant bacteria were screened for expression by analysis of their proteins after induction with 5 mM IPTG, using sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The recombinant *O. volvulus*-chitinase (rOv-chitinase) was purified from *E. coli* lysate by affinity chromatography on a nickel containing matrix (Ni-NTA resin; Quiagen) according to the manufacturer's instructions under denaturing conditions with 6 M urea and decreasing pH gradient. The recombinant protein was stepwise dialyzed against phosphate-buffered saline (PBS) containing 2% glycerol supplemented with Tris and NaH_2PO_4. Homogeneity of the recombinant protein was determined by SDS-PAGE.

2.2. Activity assay

Chitinase activity was measured by addition of samples (0.2–5 μg) to 2 ml reaction volumes of 5 μM 4-methylumbelliferyl-*N*,*N*,*N*-triacetylchitotrioside in 50 mM $NaPO_4$, pH 6.4 at 37 °C, according to [20]. The generation of fluorescent product was monitored with a spectral fluorescence photometer (SFM 25, Contron, Neufahrn, Germany) using 350 nm excitation wavelength and 440 nm emission. Chitinase of *Serratia marcescens* (Sigma, Deisenhofen, Germany) was a positive control, whereas an irrelevant recombinant protein of filarial origin, *A. viteae*-cystatin [21], which was expressed and purified under identical conditions, served as a negative control protein.

2.3. Animals

BALB/c mice (haplotype $H2^d$) were obtained from Charles River Laboratory (Sulzfeld, Germany). Eight-week-old mice were immunized three times in intervals of 10 days by subcutaneous injection of 25 μg rOv-chitinase dissolved in an adjuvant containing 10% Squalene, 0.4% Tween 80 and 1% Pluronic L 121 in PBS as described by [22].

2.4. Cell proliferation assays

Cultures of spleen cells of mice were set up in duplicates in 96-well round bottom microtiter plates in a 200 μl volume at a density of 4×10^5 cells per well. The culture medium consisted of RPMI 1640 (Gibco, Grand Island,

NY) supplemented with 25 nM Hepes (Gibco), 5×10^{-5} M 2-mercaptoethanol (Sigma) and 5% FCS (Sigma). The rOv-chitinase or chitinase-peptides were added to the wells at the start of the cultures. Cultures were kept in a humidified atmosphere at 37 °C containing 5% CO_2 for 5 days. The final protein concentration was 10 μg per well for rOv-chitinase and chitinase-peptides. Negative controls of cells alone in medium and positive controls of cells incubated with phytohemagglutinin (PHA, 2 μg/ml; Gibco) were included in each assay. For the final 18 h, 0.5 μCi of ^{3}H-labeled thymidine (ICN, Eschwege, Germany) was added to each well. Incorporated radioactivity was quantified by scintillation counting with a Trilux 1450 counter (Wallac, Turku, Finland). Results were expressed as a mean of duplicates in net counts per minute (cpm). Net cpm were cpm of stimulated cultures minus cpm of unstimulated control cultures.

2.5. Synthetic peptides

The 122 overlapping 16mer peptides with 12 aa overlaps were prepared according to standard spot synthesis protocols [23] using a spot synthesizer (Abimed GmbH, Langenfeld, Germany) as previously described [24,25]. The final C-terminal 12 aa were repeated in the N-terminal 12 aa of the following peptide. For generation of sequence files, the software DIGEN (Jerini Bio Tools GmbH, Berlin, Germany) was applied. After synthesis and cleavage of the protection groups, the peptides were released from the cellulose support as carboxamides by ammonia vapor. While still adsorbed to the membrane, the spots (0.25 cm^2) were punched out and transferred to 96-well microtiter plates. Each spot contained roughly 50 nmol of peptide. Every 20th peptide was synthesized in duplicate and analyzed by HPLC–MS (HPLC: HP1100, Hewlett-Packard, Waldbronn, Germany; MS: ESI LCQ, Finnigan MAT, Bremen, Germany) demonstrating purities of >70%.

2.6. Prediction of T cell epitopes

T cell epitopes of the *O. volvulus*-chitinase were comparatively predicted with three algorithms. The algorithm designed by [26] identifies a sequence pattern of 5 aa in length. This pattern consists of a charged aa or glycine in the first position, followed by two hydrophobic aa. The fourth position is occupied by a proline or a hydrophobic aa while a polar aa or glycine follows in the last position. According to [27], the grouping of aa follows the patterns suggested by [28]. Since T cell stimulation in the context of MHC Class II molecules requires peptides of 12–24 aa length [29,30], the identified 5 aa sequences of *O. volvulus*-chitinase were expanded N-terminally and C-terminally to yield peptides of 15–16 aa, thus representing parts of the protein sequence with the Rothbard and Taylor motif in its center.

Allele-specific peptide motifs [30] are defined by the positions of aa which interact with specific residues within the MHC Class II-binding grooves ("anchor positions"). The $H2^{d}$ peptide motif has been reported previously [31]; epitope prediction is available on the web page http://www.uni-tuebingen.de/uni/kxi using the database SYFPEITHI [32]. The prediction results in 15mer peptides, with nine core aa supposed to be bound within the binding cleft and three flanking residues at either terminus.

The third algorithm is that of Humphreys and colleagues. It is based on the motifs of [30] with modifications for chemical equivalence of side chains [33,34]. The protocol for determinations of such designer peptides (here to be E^{d}-presented) is as follows.

1. Given, the aa sequence of an antigenic protein.
2. Identify the set of K, R, and H residues in the sequence.
3. Identify the subset of #2, whenever the aa in the position −8 or −7 with respect to each K or R is a member of the group F, Y, W, I, L, V, and M. The N-terminal of such scored hydrophobic aa becomes the putative 'P1 pocket' residue of the motif. The index K, R, or H residue of the motif is called the putative 'P9 pocket' residue, regardless of its occurrence in either the eighth or ninth sequence position.
4. Identify the subset of #3 whenever the aa in the putative 'P4 pocket' position (the fourth sequence position in the motif) is a member of the group K, R, H, and I.
5. Identify the subset of #3 whenever the aa in the putative 'P6 pocket' position (the sixth sequence position in the motif) is a member of the group I, L, and V.
6. Identify the common subset of #4 and #5.
7. Synthesize peptides, according to resources, in the following priority: sets #6, #4, #5, #3, provided that for each peptide, 8 or 9 aa of the primary sequence are included from the P1 pocket residue through the P9 pocket residue, and furthermore, that such cores are extended 2 aa according to the native sequence in both N-terminal and C-terminal directions. Thus, each synthetic peptide is 12 or 13 aa.

2.7. Specificity of T cell epitope prediction

The predictive power of the algorithms was characterized as the ratio of experimentally confirmed epitopes/total number of predicted epitopes.

3. Results

3.1. Expression of recombinant O. volvulus-chitinase

E. coli transformed with pQE30 plasmids containing the *O. volvulus*-chitinase cDNA produced a 56 kDa protein upon induction, which accounted for about 20% of the bacterial biomass. Purification by nickel affinity chromatography under reducing conditions and subsequent renaturation

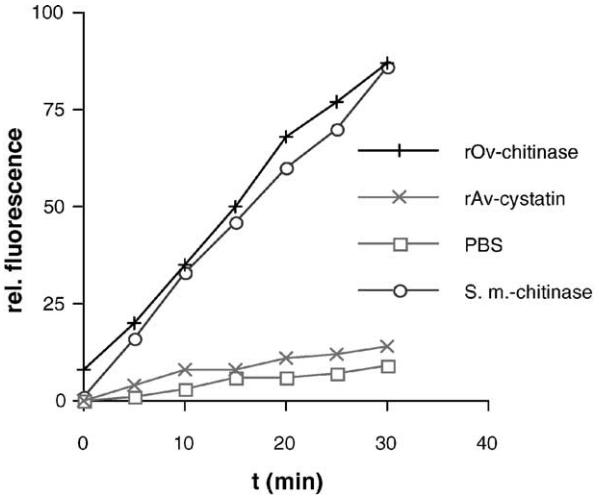

Fig. 1. Enzymatic activity of recombinant *O. volvulus*-chitinase (rOv-chitinase). Release of 4-methylumbelliferone from GlucNac3-MeU in the presence of rOv-chitinase (0.5 μg/ml), chitinase of *S. marcescens* (Sm-chitinase, 0.2 μg/ml), the recombinant control protein Av17 (rAv-cystatin, 0.5 μg/ml) or phosphate-buffered saline (PBS).

by stepwise dialysis yielded a buffer soluble recombinant *O. volvulus*-chitinase which ran as a homogenous band in SDS-PAGE with some lower molecular weight bands. These bands proved to be proteolytic degradation fragments which were recognized by mouse sera against rOv-chitinase in immunoblots (data not shown). The purified protein hydrolyzed the substrate $GlucNac_3$-MeU, similarly to a chitinase of the bacterium *S. marcescens*, whereas a recombinant control protein was inactive (Fig. 1). These results confirmed expression and purification of rOv-chitinase as an enzymatically active protein.

3.2. Mapping of T cell epitopes with a library of overlapping peptides

To map T cell epitopes of *O. volvulus*-chitinase, a panel of 122 overlapping 16mer peptides with 12 aa overlaps (pepscan) spanning the entire deduced sequence of the protein was synthesized and tested. The proliferative response to each of these pepscan peptides was assayed with spleen cells of BALB/c mice which had been immunized with rOv-chitinase. Marked, peptide-specific differences in proliferative response were observed (Fig. 2), while splenocytes from unimmunized mice were non-responsive.

The analysis of the library of overlapping peptides revealed 14 stimulatory regions which reproducibly induced proliferative responses (>1000 net cpm). The peptides derived from the N-terminal signal sequence did not stimulate proliferation of splenocytes, while many peptides from the adjacent catalytic domain (aa 21–391) were stimulatory. Interestingly, in the C-terminal, chitin-binding domain (aa 392–497) only one peptide induced proliferation (Fig. 2). The intensity of proliferation in this assay varied substantially among individual peptides, reaching up to 4600 cpm with certain peptides while other peptides consistently induced much weaker responses. In some cases, a strongly stimulating peptide was flanked by less stimulatory peptides (e.g. peptides between aa 217 and 244). Such a setting could reflect overlapping, distinct epitopes and/or the effect of adjacent sequences on the activity of one dominant epitope. In some cases one could suggest a core epitope, i.e. the aa common to a series of peptides. For example, the peptides aa 217–232, aa 221–236, aa 225–240, all of which induced proliferation of >2500 cpm, have a common core region—LHAKLHPT. In other segments, non-overlapping adjacent peptides were stimulatory (e.g. the peptides in the region between aa 349 and 379), indicating the presence of several adjacent epitopes.

3.3. Selection of T cell epitopes by algorithms

Application of T cell epitope-predicting algorithms of [26], of [32], and of Humphreys (see Section 2) to the deduced aa sequence of the *O. volvulus*-chitinase produced three sets of peptides, some of which overlapped among the predicted sets. The Rothbard and Taylor algorithm predicted 24 motifs to be T cell-recognized epitopes (Table 1, Fig. 3). The Rammensee algorithm predicted 10 H2-E^d motifs, all of which were located within the catalytic domain (aa 21–391; Table 1, Fig. 3). All except one of the Rammensee patterns overlapped with at least one Rothbard and Taylor motif. The algorithm of Humphreys predicted 13 stretches with potential T cell antigenicity (Table 1, Fig. 3). These stretches were located within the catalytic domain and within the chitin-binding domain. Each stretch overlapped with at least one Rothbard and Taylor motif, and some of the stretches also co-localized with a Rammensee motif.

The epitopes predicted by the algorithms clustered and overlapped in five regions of the protein (Fig. 3). The five putative T cell stimulating domains, which were predicted with all three algorithms (listed in Table 2) were located within the catalytic domain. The first sequence (aa 39–52) was recognized by the algorithms of Rammensee and Humphreys as a contiguous stretch, while the Rothbard and Taylor prediction identified two noncontiguous epitopes within this region. The second sequence (aa 222–236), detected by the algorithm of Rammensee as one contiguous stretch, contained one Rothbard and Taylor motif, while the prediction of Humphreys recognized two stretches with an overlap of 8 aa. The third domain (aa 247–264) contained two overlapping Rammensee motifs, while it was part of two Rothbard and Taylor motifs and of two Humphreys motifs, respectively. The fourth domain (aa 301–314) consisted of one Rammensee motif, a nearly identical Humphreys motif and two adjacent Rothbard and Taylor motifs. Region 5 (aa 341–354) contained three overlapping or adjacent Rothbard and Taylor motifs, one motif according to Rammensee and a Humphreys motif.

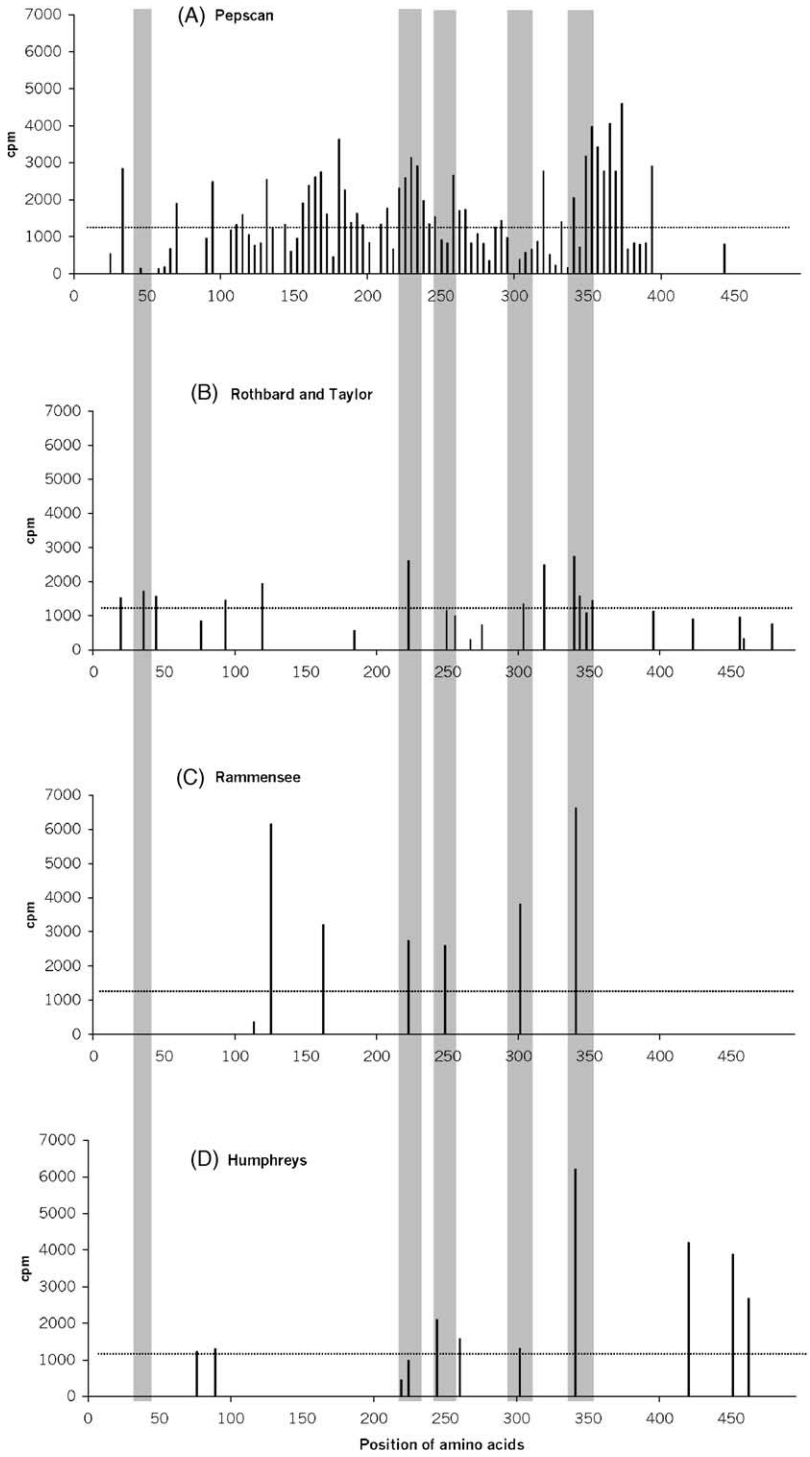

Fig. 2. Proliferative responses of murine spleen cells after re-stimulation with synthetic peptides of *O. volvulus*-chitinase; representative experiment: (A) stimulation with overlapping peptides (pepscan); (B) stimulation with peptides predicted by the Rothbard and Taylor algorithm; (C) stimulation with peptides predicted by Rammensee algorithm; and (D) stimulation with peptides predicted by Humphreys algorithm. The *x*-axis represents the amino acid sequence of rOv-chitinase running from the N-terminus (left) to the C-terminus (right). T cell epitopes are marked with left justification. Bars represent stimulatory peptides, dotted lines represent the threshold of 1000 cpm. Shaded areas represent the five T cell stimulatory domains determined by predictions and experiments.

Table 1
Localization of predicted T-cell epitopes within the *O. volvulus*-chitinase sequence and induced proliferative response in comparison with pepscan peptides

Position of peptides within the aa sequence	Rothbard and Taylor peptides	Proliferative response
19–34	1. AYGYV**RGCYY**TNWAQY	+
35–48	2. RQGEG**KFLPE**DIPKG	+
44–58	3. EDIPK**GLCTH**ILYAF	+
76–90	4. DTNWS**KGMYS**RVTKL	–
93–107	5. NDPEM**KILLS**YGGYN	+
119–134	6. RAEKR**KHFIK**SAIAFL	+
184–198	7. AVSAG**KHTID**QSYNV	–
222–237	8. NVDLHA**KLHPT**KGETS	++
249–263	9. NYWLS**KGMPK**QKIII	+
255–269	10. GMPKQ**KIIIG**IPTYG	+
266–280	11. PTYGR**GWTLR**DSSKT	–
274–288	12. LRDSS**KTTIG**AEGIS	–
295–309	13. TNPAG**GTAAY**WEICK	–
304–318	14. YWEIC**KYLKE**GGKET	+
318–332	15. TIDEQ**GVGAC**MVQGS	++
339–353	16. NEETI**RMKMR**WLKEK	++
343–357	17. IRMKM**RWLKE**KGYGG	+
348–362	18. RWLKE**KGYGG**AFIWT	+
352–366	19. EKGYG**GAFIW**TLDFD	+
395–419	20. TTRSL**RTTIT**QSSTI	–
423–437	21. ITKNN**KIKTT**TIAVE	–
456–470	22. HPND**CHLFI**HCAHDH	–
459–474	23. CHLFI**HCAHD**HPYVK	–
479–494	24. TFFND**KIKVC**DHFGE	–
Position of peptides within the aa sequence	**Rammensee peptides**	**Proliferative response**
38–52	1. EGKFLPEDIPKGLCT	–
113–127	2. FTAIRNRAEKRKHFI	–
125–139	3. HFIKSAIAFLRKNKF	+++
162–176	4. EMKVAFVEEAKKSDS	+++
222–236	5. NVDLHAKLHPTKGET	++
247–261	6. AANYWLSKGMPKQKI	–
248–262	7. ANYWLSKGMPKQKII	++
269–283	8. GRGWTLRDSSKTTIG	–
301–315	9. TAAYWEICKYLKEGG	+++
340–354	10. EETIRMKMRWLKEKG	+++
Position of peptides within the aa sequence	**Humphreys peptides**	**Proliferative response**
39–51	1. GKFLPEDIPKGLC	–
76–88	2. DTNWSKGMYSRVT	+
81–93	3. KGMYSRVTKLKEN	–
89–101	4. KLKENDPEMKILL	+
219–231	5. WEMNVDLHAKLHP	–
224–236	6. DLHAKLHPTKGET	–
244–256	7. TEFAANYWLSKGM	++
260–272	8. KIIIGIPTYGRGW	+
302–314	9. AAYWEICKYLKEG	+
341–353	10. ETIRMKMRWLKEK	+++
420–432	11. ASEITKNNKIKTT	+++
451–463	12. FGLFRHPNDCHLF	+++
462–474	13. LFIHCAHDHPYVK	++

The magnitude of the proliferative response is indicated as follows: (+) net proliferation between 1000 and 2000 cpm; (++) net proliferation between 2000 and 3000 cpm; (+++) net proliferation >3000 cpm. The core amino acids of the Rothbard and Taylor motifs are in bold characters.

3.4. Proliferative T cell analysis of designer peptides containing algorithm-predicted T cell epitopes

The proliferative response of splenic lymphocytes of mice immunized with rOv-chitinase was determined with each of the designer peptides. A representative experiment is shown in Fig. 2. A stimulation of more than 1000 net cpm was taken as the threshold for a positive value. The proliferative responses in cpm were generally low but reproducible (Fig. 2, Table 1). Of the 24 Rothbard and Taylor peptides, 14 stimulated T cells (58% confirmed; specificity of 0.58). Eleven peptides had net proliferation values between 1000

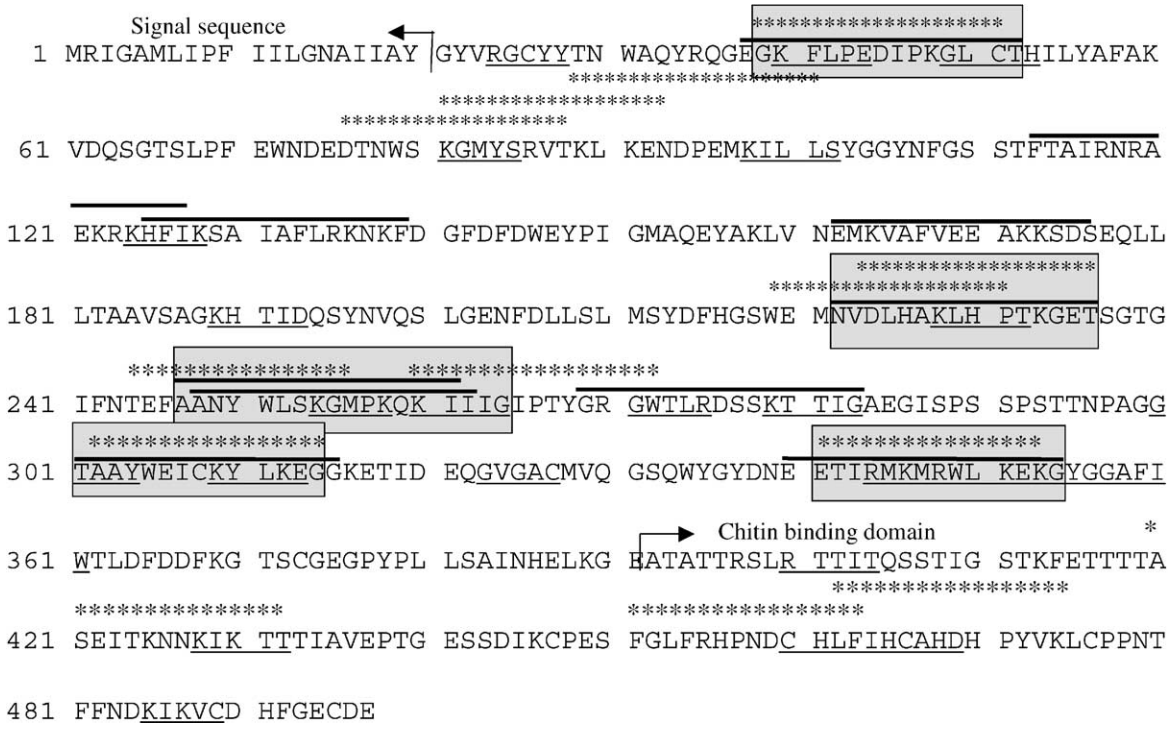

Fig. 3. Deduced amino acid sequence of *O. volvulus*-chitinase with predicted T-cell epitopes. The predicted Rothbard and Taylor motifs are underlined. Rammensee motifs (for $H2^d$) are overlined in bold. Humphreys motifs are marked with asterisks above the sequence. The five T cell stimulatory regions with clustered and overlapping motifs are boxed.

and 2000 cpm; the other three produced >2000 cpm, with maximal counts of 2740 cpm. Of the 10 Rammensee motifs, 6 stimulated T cells (60% confirmed; specificity of 0.6). All six induced strong proliferative responses of >2000 cpm with maximal counts of 6619 cpm. Out of the 13 T cell stimulating stretches predicted by Humphreys algorithm, 9 synthetic peptides re-stimulated T cells (69% confirmed; specificity of 0.69). Four peptides induced proliferation between 1000 and 2000 cpm above background, while strong proliferative responses of >2000 net cpm were obtained after stimulation with five other peptides (maximal counts 6216 cpm).

The best concordance between predicted and biologically active designer epitopes was found in the five domains with clustered epitopes discussed previously. Sequences of aa

Table 2
Proliferative responses of murine splenocytes induced by pepscan peptides and designer peptides of five T cell stimulatory domains within the chitinase of *O. volvulus*

Position of T cell stimulatory domain within the aa sequence	Rothbard and Taylor peptides	Proliferative response to Rothbard and Taylor peptides	Rammensee peptides	Proliferative response to Rammensee peptides	Humphreys peptides	Proliferative response to Humphreys peptides
aa 39–52	RQGEG**KFLPE**DIPKG	+	EGKFLPEDIPKGLCT	–	GKFLPEDIPKGLC	–
	EDIPK**GLCTH**ILYAF	+				
aa 222–236	NVDLHA**KLHPT**KGETS	++	NVDLHAKLHPTKGET	++	WEMNVDLHAKLHP	–
					DLHAKLHPTKGET	–
aa 247–264	NYWLS**KGMPK**QKIII	+	AANYWLSKGMPKQKI	–	TEFAANYWLSKGM	++
	GMPKQ**KIIIG**IPTYG	+	ANYWLSKGMPKQKII	++	KIIIGIPTYGRGW	+
aa 301–314	TNPAG**GTAAY**WEICK	–	TAAYWEICKYLKEGG	+++	AAYWEICKYLKEG	+
	YWEIC**KYLKE**GGKET	+				
aa 341–354	NEETI**RMKMR**WLKEK	++	EETIRMKMRWLKEKG	+++	ETIRMKMRWLKEK	+++
	IRMKM**RWLKE**KGYGG	+				
	RWLKE**KGYGG**AFIWT	+				

The magnitude of the proliferative response is indicated as follows: (+) net proliferation between 1000 and 2000 cpm; (++) net proliferation between 2000 and 3000 cpm; (+++) net proliferation >3000 cpm. The core amino acids of the Rothbard and Taylor motifs are in bold characters.

39–52 were stimulatory with Rothbard and Taylor peptides. Sequences of aa 222–236, aa 247–264, aa 301–314 and aa 341–354 were also stimulatory with peptides predicted by the algorithms of Rothbard and Taylor, Rammensee, or Humphreys (Fig. 2, Table 2).

Some of the stimulatory designer peptides matched well with stimulatory pepscan peptides (see Fig. 1). Such a correspondence occurred in the previously mentioned five T cell stimulatory regions of the protein (see Fig. 3). In other cases, designer peptides and pepscan peptides representing similar stretches of the protein did not have the same stimulatory capacity. This pertained particularly to the C-terminal chitin-binding domain (aa 392–497), where three designer peptides identified with the Humphreys algorithms induced proliferative responses, while only a single pepscan peptide was stimulatory.

4. Discussion

Adjuvant peptide vaccines for T helper cell-recognized epitopes may enhance the potency and memory of the response to secondary immunization with other protein or DNA vaccines against pathogens or cancer [18]. In order to pursue this benefit for the development of a vaccine against onchocerciasis, we have undertaken a systematic study to identify such MHC Class II-presented peptide determinants within the chitinase of *O. volvulus*. In this report, we compare data on the identification of such epitopes with both a library of overlapping peptides and peptides based on motif analysis. The labor is justified by the importance of characterizing all T cell-recognized epitopes, for several clinically relevant reasons. First, for simplicity, one desires to create vaccines with most potent peptides, each with the widest range of MHC Class II allele presentation. However, in the event that such potent peptides are not presented in an appreciable fraction of the immunized population, then one wants to know all the peptides which might be presented. A larger "basket of peptides" might be required. We used inbred mice of the BALB/c strain (haplotype $H2^d$) in order to complete the studies in genetically defined and uniform animals. Since our study is exploratory in character and accents a comparison of methods, we concentrated on one strain. Comparisons among several strains of inbred mice can be done in the future using the procedure found to be optimal in our current experiments.

In comparing results with these two approaches, the detection limit was set at 1000 cpm. Such a low threshold has been used, because the peptides are bound to surface-exposed MHC Class II molecules of antigen presenting cells, instead of undergoing the usual pathway of antigen processing and presentation [35]. Therefore, stimulation by externally loaded peptides results in lower T cell proliferation compared to stimulation with crude antigen [36–38]. Other studies, where sensitized T cells were stimulated with synthetic peptides [39–41] used similarly low thresholds. In order to be confident of our determinations, assays were repeated up to seven times, with good concordance of the observations.

Whenever a single pepscan peptide in a series was positive, this allowed the exact mapping of an epitope. However, in runs of two to seven adjacent stimulatory peptides, clearly more than one epitope was recognized, making difficult the proposal of specific epitopes [42]. In addition, our method of analysis, with synthesis and testing of very low quantities of each of a large number of sequences, precluded dose/response testing with individual peptides. At higher concentrations, the activity of less potent sequences might be detected. Likewise, the potency of the best sequences could be better defined upon titering the response to lower concentrations of synthetic peptides. Such efforts can be undertaken with the best sequences identified here.

Many of the epitopes identified with the three predicting algorithms were biologically active. With the Rothbard and Taylor algorithm, 58% of the predicted epitopes were active in the proliferation assay. This algorithm detects short stretches of aa typical for amphipathic helices. The hydrophobic aa in such structures are typical P1 anchor residues which allow binding of peptides to MHC Class II proteins. The Rammensee algorithm had a specificity of 60%. This algorithm was empirically developed by pattern analysis of the distribution of aa in linear sequence positions of endogenously processed antigenic peptides. The algorithm of Humphreys and colleagues had a specificity of 69% in this study.

The T cell stimulatory epitopes identified with the library of overlapping peptides did not always match with those determined with the predicted algorithms. Some stimulatory pepscan epitopes were not predicted with the algorithms (e.g. the peptides covering the region between aa 169 and 184), while some biologically active, algorithm-predicted epitopes were not detected among the pepscan peptides (e.g. peptides encompassing aa 301–314). This variation probably reflects also flanking region effects [43,44]. This observation reinforces our concern at the beginning of a comprehensive effort to develop clinically effective MHC Class II-presented peptide vaccines, to search for biological activity with multiple approaches. No simplistic approach to picking epitopes by either library scanning or algorithm predicting will identify all potentially useful epitopes.

A potentially useful finding of this study was the clustering of biologically active epitopes. Similar clusterings have been observed by [45] in studies of algorithm-predicted epitopes in a *M. tuberculosis* protein. Such regions presumably reflect peptidyl segments in which multiple alignments within the MHC Class II antigenic peptide-binding cleft are possible [46]. Preference for one of the overlapping alignments leads directly to the selection of a dominant epitope. The overlapping epitopes which are selected against, thus become cryptic epitopes, i.e. recognized upon immunization of mice with peptides but not with the intact protein [47]. Which epitope is dominant in any given MHC Class II allele might vary among the clustered epitopes.

Our analysis shows that the combination of several predictive algorithms allows the identification of biologically active T cell epitopes with relative ease. A parallel use of algorithms for different MHC Class II haplotypes will allow the detection of functional T cell epitopes restricted by other MHC Class II molecules and comprehensive information on clusters of T cell epitopes recognized by a broad range of alleles can be compiled. Such protein fragments including epitopes presented by multiple MHC Class II haplotypes, termed "promiscuous" or "universal" T cell epitopes, have been defined in viral, bacterial and parasite proteins [42,48–50]. Consequently, a peptide vaccine encompassing such promiscuous epitopes will offer protection to a wider range of individuals with different HLA-DR, -DQ, -DP histotypes. Such segments are targets for DNA minigene vaccines or can be applied as multiepitope vaccines in the form of mosaic proteins. Further studies in animal models will show whether the use of such characterized T cell epitopes will significantly increase the partial protection against challenge infection so far obtained with filarial chitinases.

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