Immunization of Sheep with Phage Mimotopes against Dermatophilosis

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ABSTRACT

Random peptide libraries (RPL) displayed on the surface of filamentous bacteriophages have been extensively used as a tool to map epitopes or to identify antigenic mimics (mimotpoes) of disease-specific monoclonal antibodies or polyclonal sera. These RPL are engineered by the insertion of degenerate oligonucleotides, encoding a specific number of random amino acids, in frame with a bacteriophage gene specifying a virion surface protein. The RPL are constructed by inserting 21 to 36 random nucleotides into the gene for an appropriate coat protein in a phage that is propagated in *E. coli*. Particularly, clear mimotopes have been obtained with monoclonal antibodies and with a few polyclonal antibodies against viruses. The choice and processing of sera for the selection of the disease related mimotopes and sera to remove mimotopes reacting with ubiquitous antibodies are important in studies of RPL. We have used RPL to select mimotopes of antigens to be used for immunization against dermatophilosis. IgG from sheep protected from dermatophilosis with an enzyme preparation from *Dermatophilus congolensis*, was used to select peptides displayed on phage in the Ph.D.-7 random peptide library which contains 10^9 peptides. Phage displaying peptides that were unrelated to the mimotopes associated with protection to dermatophilosis was removed with IgG from sheep that were vaccinated with the enzyme preparation but were not protected. The IgG from the protected sheep, before vaccination, was also used for the negative selection. The selected mimotopes, those with clearly repeating motifs, were chosen and used to immunize sheep. A mixture of four phage mimotopes induced antibody to a recombinant protease from *D. congolensis*. The immunized sheep recovered more rapidly from the lesions caused by the strains when challenged with two strains of *D. congolensis*. *Iran. Biomed. J. 6 (4): 129-134, 2002*

INTRODUCTION

Dermatophilosis is a chronic or acute skin disease of animal and man caused by *Dermatophilus congolensis*. Vaccination of sheep is a desirable method for control of the disease. So far it has been partially successful because the antigens are difficult to produce in sufficient quantity from the *D. congolensis*.

An alternative is to use random peptide libraries (RPL) as a tool for the production of novel vaccines. Peptides that mimic epitopes (mimotopes) on pathogens are potential agents to induce the protective antibodies, and thus may be used to elicit protective antibodies of the same specificity in non-immune animals [1]. RPL have been pioneered by Smith and others [2-4] that enables huge populations of diverse peptides to be screened.

Specific members of these populations are selected on the basis of their binding affinity to an immobilized target. In this technique, DNA sequences encoding highly diverse libraries of peptides are fused to the 5'-end of bacteriophage coat protein genes. Following expression, these fusions are folded, assembled and exposed to the random peptides on the surface of the bacteriophage [4]. The peptides in the random library are then given the opportunity to bind to an immobilized target protein and to peptides displayed on a phage. The specific interaction with the target is selectively retained through a wash procedure. Bound phage is eluted and then submitted for additional rounds of selection after amplification in *E. coli* or for further analysis. Induction of specific immune responses by mimotopes to antigens has been demonstrated [5-7]. Galfre *et al*. [8]

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summarized their preliminary work on vaccination with mimotopes against hepatitis B. Bastien *et al.* [9] claimed to be the first group to produce a protective vaccine using a phage displayed peptide in mice.

In this paper, we investigate whether immunization of sheep with mimotopes prepared from Ph.D.™ could produce specific antibody responses and give protection against challenge with *D. congolensis* zoospores. If they were effective, the phage displayed antigens could be provided at low cost and they could be incorporated into the existing vaccine to give an improved dermatophilosis vaccine for commercial use.

MATERIALS AND METHODS

IgG preparation. IgG was prepared from the pooled sera from the protected, non-protected and pre-immune groups of sheep that were vaccinated against dermatophilosis with a vaccine based on the serine protease of *D. congolensis*. Sera were kindly donated by Dr. T.M. Ellis (Agriculture Western Australia) from an unpublished experiment that was part of a commercial contract. The procedure was carried out as described in the Ph.D.™ kit (New England, Biolabs) with IgG from the protected group for positive selection. Three culture flasks (Corning Costar Corporation, tissue culture flask, canted neck, plug lid, Cat. No. 430168-2510025) were coated with 200 μg of IgG in 2 ml of 0.1 M $NaHCO_3$, pH 8.6 for 3 rounds of biopanning and swirled repeatedly until the bottom surface of the flasks were completely wet. The following day, the solutions were poured off from each flask and the flask neck firmly slapped onto a clean paper towel to remove residual solution. Then, the flasks were washed 3 times with TBS (50 mM Tris-HCl pH 7.5, 150 mM NaCl), blocked with 50 ml of blocking buffer (0.1 M $NaHCO_3$ pH 8.6, 5 mg/ml BSA, 0.02% NaN_3) and incubated at 4°C for 1-2 h. The blocking buffer was discarded from one of the flasks (two flasks were stored at 4° C for using later) and the flask was washed 6 times with TBST (TBS + 0.1% (v/v) Tween-20).

Phage selection. Ten μl of the library (2×10^{12} phage) was diluted in 2 ml of TBST and pipetted into coated flask and rocked gently at room temperature for 60 min. Non-binding phage was discarded by pouring off and slapping flask neck down onto a clean paper towel. The flask was washed 10 times with TBST followed 2 times with only TBS using a clean section of paper towel each time to prevent cross-contamination.

To elute bound phage, 2 ml of acidic buffer (0.2 M glycine pH 2.2, 1 mg/ml BSA) was added to the flask for 10 min at RT on a rocker to elute additional phage peptides. The eluate was decanted and neutralized with 1M Tris-HCl (pH 9.3) and then was amplified (as described in the Ph.D.™ kit). After the amplification, the selection procedure was repeated two times to enrich the concentration of the phage selected by the antibody of interest and eluted with acidic buffer. The amplified eluate from the first round was used as phage for the second flask and then amplified eluate from the second round was used as phage for the third flask.

To remove antigenic peptides, which would react with commonly occurring antibodies, flasks were coated with IgG from the pre-immune and from the non-protected groups as negative selection. The amplified eluate phage from the third round was added to the first blocked pre-immune flask and incubated for 1 h at RT on rocker. The contents of the first flask were transferred into the second flask and then incubated as above. The procedure was also carried out with 2 flasks of non-protected IgG for negative selection. The phage was plated and from the surviving clones, 49 clones were chosen randomly for DNA sequencing.

PCR sequencing. This technique was carried out to amplify DNA form Ph.D.™ clones. Cycle sequencing was carried out using the Dye Terminator Cycle Sequencing Reaction (Perkin-Elmer). For 20 μl of each reaction, 3.2 pmole of the primer (5'-CCC TCA TAG TTA GCG TAA CG-3') which was supplied with the Ph.D.™ kit, 8 μl of Terminator Ready Reaction Mix and 3.8 of sterile distilled water were mixed and then 5 μl (~100 ng) of phage DNA as template was used. The amplification was preceded for 25 cycles in a DNA thermal cycler (Perkin-Elmer). Each cycle involved a 96°C-denaturing step for 10 s followed by 5 s annealing temperature at 50°C and finally the polymerization step at 60°C for 4 min. The polymerisation time for the last cycle was carried out for 10 min to obtain the full length of amplified products. Each extension product was run on a 1% (w/v) agarose gel to ensure that there was sufficient DNA for sequencing. Following cycle sequencing, excess terminators were removed by ethanol precipitation protocol 1 Dye Terminator Cycle Sequencing Reaction and the purified samples were electrophoresed on a 3073A automatic DNA sequencer.

Nucleotide sequences obtained through the automatic DNA sequencer were analysed using the sequence editor program SeqEd[TM] version 1.0.3 (Applied Biosystems Inc) on an Apple Macintosh computer. The sequences were aligned with each other. The sites of the random peptides inserted were located using the sequence editor program. The inserts were 21 base pairs (bp) long for Ph.D.[TM] clones. These nucleotide sequences were translated into heptamer amino acid sequences by the sequence editor program. Hepta sequences were analysed using a number of different sequence analysis programs that were provided by Australian National Genomic Information Services (ANGIS). From the total clones, 28 with sufficient DNA were compared by PILEUP computer program that generates a figure showing similarity between peptides [10].

Immunization and protection assays. For immunization with phage mimotopes, phage peptides (pp) 24, pp25, pp30, and pp42 (Fig. 1) were each amplified [11] and mixed together. The immunization experiment was done in attempts to protect sheep against dermatophilosis. 24 Merino hogget ewes with no evidence of dermatophilosis and origination from a flock with any history of dermatophilosis were randomly allocated to 2 groups of 6 sheep. Blood samples were collected and then each sheep in group 1 was immunized with 2×10^{14} pfu in 1 ml PBS ($\sim$ 1 mg protein). Montanide ISA206 (as an adjuvant) was mixed with an equal volume of antigen to form a water-in-oil emulsion giving a dose of 2 ml that was inoculated subcutaneously into one site in the neck region of each sheep. Sheep in group 2 were immunized with adjuvant in PBS. Twenty-eight days after the first injection, the sheep were re-immunized as described above. Twenty-one days after the second immunization each sheep was challenged with 400 μl zoospore suspension of Ag (Agriculture) and MB (Mount Barker) of *D. congolensis*. After challenge, lesions were scored 7, 14 and 21 days later using the following scoring system [12, 13].

ELISA. To screen the activity of antibody against the serine protease, ELISA was used according to reference [14] and also the manual of the Chiron Mimotopes Peptide Systems. Assays were used in the 96-well micro-titer plates (Maxisorp). All the solutions were used at 100 μl per well, except the blocking solution that was 300 μl. The wells were washed 4 times with PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4, pH

7.2) containing 0.1% v/v of Tween-20. Each well was coated with 5 μg of protein in 100 μl of 0.1 M $NaHCO_3$ and the plates were incubated overnight at 37°C. The following day, after washing, the wells were blocked with 300 μl of 2% (w/v) FCS in PBS for 1-2 h at RT. Diluted sera were added and after 1 h, 1:2000 dilution of the secondary antibody (anti-goat IgG) in blocking solution which was conjugated to horseradish peroxidase was added to the plates. After 1 h, 100 μl of 0.4 M substrate ABTS was added. The absorbance was read in a dual wavelength at 405_{nm} against a reference wavelength of 495_{nm} in a Bio-Rad ELISA plate reader (Bio-Rad).
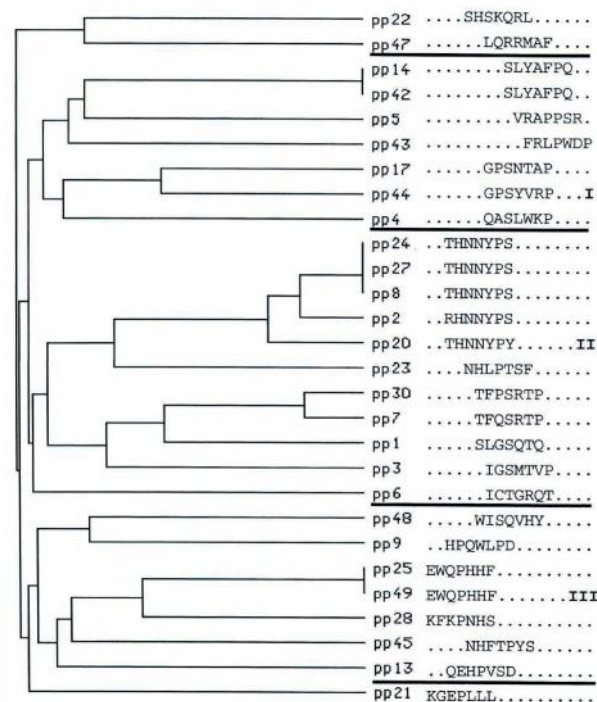

Fig. 1. PILEUP of peptides selected from the Ph.D.[TM] RPL with IgG from protected group and which survived negative selection. Peptides were assigned to three families (I, II and III). The pp indicates phage peptide.

RESULTS

IgG was separated from pooled sera of sheep from protected, non-protected and pre-immune groups. After selection of phage with protected, pre-immune and non-protected IgG, clones, eluted with acidic buffer, were chosen from the plate cultured with less than 100 plaques. A total of 49 clones were isolated from the Ph.D.[TM] RPL for DNA purification. Following quantification of phage DNA, 28 of those clones produced sufficient DNA for sequencing. Peptides selected with IgG from the

protected group from the Ph.D.™ RPL are shown in Figure 1. PILEUP showed that the peptides could be assigned into 3 major families (Fig. 1). Two peptides, pp14 and pp42 were identical in the first family SLYAFPQ while pp17, pp44 and pp4 had some similarity. Peptides pp24, pp27 and pp8 were identical in the second family THNNYPS, peptides pp2 and pp20 had some similarity to those peptides HNNYP. Also in the second family peptides pp30 and pp7 had similarity TF*SRTP. In the third family, peptides pp25 and pp49 were identical EWQPHHF whereas, pp28 and pp45 had some similarity with these peptides. When sheep were immunized with phage mimotopes, they produced little antibody to MB serine protease by day 28 after first immunization. However, there was a marked increase in the production of antibody to MB serine protease at day 49 that was 21 days after the second immunization. In 5 out of 6 sheep, which were immunized with phage mimotopes (Fig. 2) at day 49 but not at day 28, there was a significant difference from the control group ($P<0.01$). In the
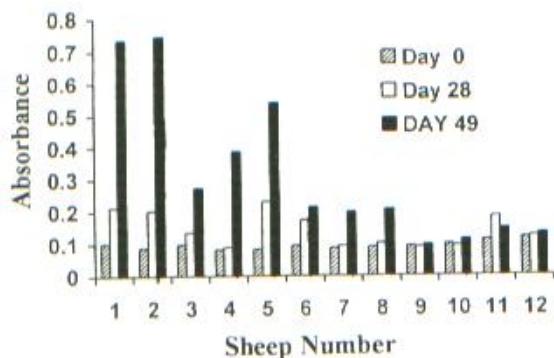

Fig. 2. Antibody to MB protease by ELISA. Numbers 1-6, represent sheep immunized with phage mimotopes. Numbers 7-12 were used as the control, which received only adjuvant. Day 0, 28 and 49 indicate per-immune, 4 weeks after first immunization and 3 weeks after second immunization respectively with a 1/200 dilution of sera. Each serum was diluted 1/200 in 2% FCS/PBS for duplicate determination by ELISA.

group immunized with phage mimotopes (Fig. 3A), there was no difference between the control and phage in the reduction of lesion scores 14 days after challenge, but there was a significant difference from the control group in the decrease in lesion score at 21 days following challenge with the W14 strain of *D. congolensis* ($P<0.001$). There was no difference between the control and phage (Fig. 3B) in the reduction of lesion scores 14 and 21 days

following challenge with the more virulent MB strain, although the immunized sheep recovered more rapidly from the lesions at day 21 following challenge with this strain.
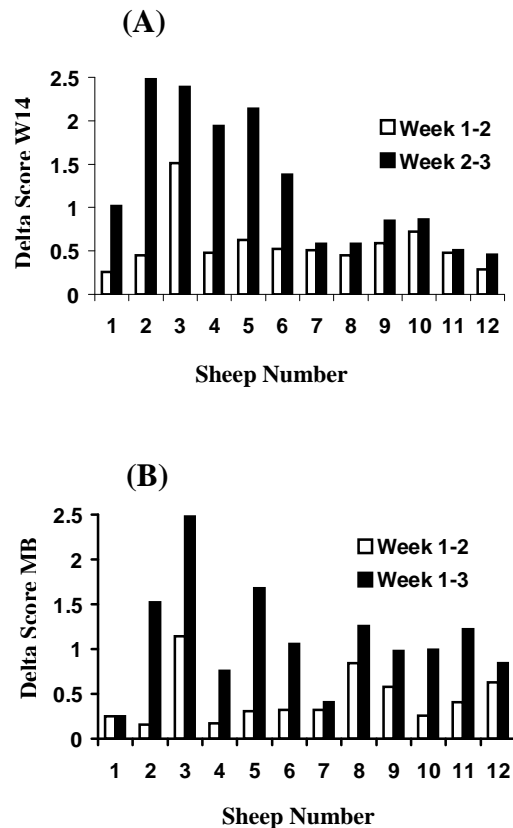

Fig. 3. Change in scores was measured between weeks 1 and 2 and weeks 1 and 3 in sheep No 1-6 which were challenged with *D. congolensis* W14 following immunization with phage mimotopes. Sheep No 7-12, which were used as controls, received only the adjuvant.

DISCUSSION

In choosing the peptides for immunization, we assumed that they are associated with protection with mimotopes of epitopes in serine protease, as the sheep had been vaccinated with an enzyme preparation enriched in serine protease. We decided not to use all the isolated peptides, because if this were done, the amount of carrier protein from the phage coat would be high relative to the individual peptides. The problem was the selection of peptides for immunization. Thus, peptides pp25, pp30, pp24, and pp42 were chosen to represent families I, II, III respectively in Figure 1. Because of the higher activity of serine protease in the virulent strain of *D. congolensis*, Ellis [15] considered it a prime candidate for inclusion in a vaccine. The results

presented in this paper showed a need to reevaluate the inclusion of protease in vaccines against *D. congolensis*. The reason for reevaluation of Ellis's conclusion is the finding that immunization with phage mimotopes resembling serine protease caused an improved resolution in the lesions with one strain of *D. congolensis*. As these mimotopes induced, in two of the sheep, high titer antibodies to serine protease suggested a direct link between an immune response to serine protease and resolution of the lesion. However, in this experiment there was no correlation between the rate of resolution of lesion and the level of antibody to serine protease in any of the sheep. This would suggest that IgG is not directly involved in resolution of the lesions but is a marker showing that an immune response is in progress.

The results reported in this paper provide the first demonstration of induction of a specific immune response against natural proteins and at least some beneficial effect after vaccination of large animals with mimotopes selected from RPL. In Italy, experiments are in progress with chimpanzees to prepare a vaccine containing phage mimotopes to human hepatitis C virus, but no details have been published (*A. Nicosia* and *P. Angeletti*, Institute for Research on Molecular Biology, Rome, personal communication). Filamentous bacteriophages displaying foreign peptide epitopes are strongly antigenic when administered parenterally [6, 16]. Delmastro *et al*. [17] showed that mimotopes are immunogenic when delivered orally and intranasal administration appears to be an effective route of delivery, resulting in the induction of high titers of specific antibodies, both systemically and in mucosal secretions. It has been reported the development of a strategy to identify disease-specific mimotopes from phage displaying random peptide libraries using sera from the selected patients [6]. It has been shown that this approach to identify potential vaccines does not require utilizing the original pathogen or antigens derived from it [5]. In a study, Bastein *et al*. [9] reported that once a pathogen-specific phage recombinant displaying a putative protective epitope/ mimotope is selected from a random peptide library, it can be directly used in protective assays. As mimotopes can be selected, analyzed and prepared in quantity much more quickly than conventional or recombinant antigens, this technology should have considerable potential for preparing novel vaccines for other diseases.

To further clarify the role of the immune response to *D. congolensis* in prevention and resolution of the lesions, additional experiments were carried out. Mimotopes of epitopes in serine protease, which induce a protective immune response, could be produced more economically than either native serine protease.

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