VACCINATION TRIALS IN SHEEP AGAINST *CHRYSOMYA BEZZIANA* LARVAE USING THE RECOMBINANT PERITROPHIN ANTIGENS Cb15, Cb42 AND Cb48

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ABSTRAK

SUKARSIH, SUTIJONO PARTOUTOMO, GENE WIJFFELS, TONY VUOCOLO, dan PETER WILLADSEN. 2000. Percobaan vaksinasi pada domba terhadap larva *Chrysomya bezziana* dengan menggunakan antigen peritrofin rekombinan Cb15, Cb42 dan Cb48. *Jurnal Ilmu Ternak dan Veteriner (Edisi Khusus)* 5 (3): 192-196.

Uji efikasi beberapa bentuk protein membran peritrofik rekombinan dari lalat *screwworm Chrysomya bezziana* telah dilakukan secara *in vitro* dan *in vivo,* yaitu sebagai antigen pada vaksinasi untuk mencegah serangan larva terhadap jaringan tubuh domba. Protein tersebut ialah Cb15 dan Cb42 yang diekspresikan pada *Escherichia coli* dan Cb48 yang diekspresikan pada *Escherichia coli* dan *Pichia pastoris*. Hasil uji *in vitro* menunjukkan bahwa perubahan bobot dan daya tahan larva yang dipelihara dalam medium serum domba yang divaksinasi tidak berbeda dengan larva yang dipelihara dalam medium serum kontrol. Cb48 dari *Chrysomya bezziana* mempunyai derajat kesamaan sekuensi yang signifikan dengan antigen PM48 dari *Lucilia cuprina*. Pemberian makan dengan serum anti-Cb48 pada larva *Lucilia cuprina* mengakibatkan penurunan bobot badan yang berbeda nyata secara statistik dibandingkan dengan kontrol meskipun perbedaan tersebut kecil, sama halnya dengan pemberian serum anti-PM48. Pada uji *in vivo*, vaksinasi dengan Cb15 dan Cb42 yang diekspresikan pada *Escherichia coli* dan Cb48 yang diekspresikan pada *Pichia pastoris* menunjukkan kenaikan bobot larva yang sedikit lebih tinggi, dan daya tahan hidup sama atau lebih tinggi dibandingkan dengan pada domba kontrol. Hasil observasi didiskusikan dalam artikel ini.

Kata kunci: *Chrysomya bezziana*, antigen recombinan, peritrofin, vaksinasi

ABSTRACT

SUKARSIH, SUTIJONO PARTOUTOMO, GENE WIJFFELS, TONY VUOCOLO, and PETER WILLADSEN. 2000. Vaccination trials in sheep against *Chrysomya bezziana* larvae using the recombinant peritrophin antigens Cb15, Cb42 and Cb48. *Jurnal Ilmu Ternak dan Veteriner (Edisi Khusus)* 5 (3): 192-196.

Recombinant forms of a number of peritrophic membrane proteins from the screwworm fly *Chrysomya bezziana* have been assessed *in vitro* and *in vivo* for their efficacy as antigens in vaccination against the tissue-invasive, larval form of the parasite. The proteins included Cb15 and Cb42 expressed in *Escherichia coli* and Cb48 expressed in both *Escherichia coli* and *Pichia pastoris*. In all cases, the *in vitro* assays of larval growth on serum from vaccinated sheep failed to show inhibition of larval weight gain or any detrimental effect on larval survival relative to controls. *Chrysomya bezziana* Cb48 has a significant degree of sequence identity with the antigen PM48 from *Lucilia cuprina.* Feeding *Lucilia cuprina* larvae on antisera to Cb48 induced a small but statistically significant reduction in weight gain, as does feeding on antisera to PM48. *In vivo*, larvae feeding on sheep vaccinated with *Escherichia coli*-expressed Cb15 and Cb42 and *Pichia pastoris*-expressed Cb48 showed marginally greater weight gain and survival which was equal to or greater than that on non-vaccinated sheep. The significance of these observations is discussed.

Key words: *Chrysomya bezziana*, recombinant antigen, peritrophin, vaccination

INTRODUCTION

Studies with the larvae of the myiasis fly *L. cuprina* have shown that vaccination of sheep with a number of intrinsic membrane glycoproteins from the peritrophic membrane of a larvae, namely the peritrophins, demonstated an immune response which inhibits subsequent growth of feeding larvae (EAST *et al.*, 1993). The mechanism of this inhibition appears to be the binding of antibody to the peritrophins in the peritrophic matrix, which in turn leads to blockage of the pores of the peritrophic membrane and interference with normal feeding and digestive processes (CASU *et al.*, 1997; TELLAM and EISEMANN, 1998). Consistent

with this model is the fact that the peritrophins from *L. cuprina* are of relatively high abundance in the peritrophic membrane (ELVIN *et al.*, 1996). Vaccination trials with whole peritrophic membrane from *C. bezziana* (SUKARSIH *et al.*, 2000) as well as fractions of the peritrophic membrane (RIDING *et al.*, 2000) has suggested that a similar mechanism might operate with the screwworm fly. Certainly, larvae feeding on sheep vaccinated with whole peritrophic membrane or on the serum from sheep vaccinated with peritrophic membrane or fractions of the peritrophic membrane, show markedly reduced growth and, particularly *in vitro*, increased mortality.

Identification of the antigens responsible for the effects seen with whole peritrophic membrane is potentially a difficult and laborious process. Vaccination with a number of fractions from peritrophic membrane however suggests that relatively less complex groups of proteins can produce similar effects (RIDING *et al.*, 2000). Results with *L. cuprina* would suggest that it is the ability of antibody to bind to an antigen appropriately located on the peritrophic membrane that is critical, rather than the specific nature of that antigen itself. If this model is tentatively assumed to apply to *C. bezziana*, then it would be reasonable to assess the most abundant protein species from the active fractions of the peritrophic membrane as vaccine antigens.

Such evaluation of individual proteins from fractions of the peritrophic membrane is difficult. Although the proportion of particular protein species within the membrane may be relatively high, the absolute amount of protein that can be recovered from peritrophic membrane is small, and, for vaccination trials, limiting. To overcome this, the proteins are first identified and characterised by the limited amino acid sequence information that can be obtained from microgram quantities of the protein. This in turn is used to identify the gene coding for the protein in *C. bezziana,* leading to subsequent expression as a recombinant protein in a form suitable for vaccination trials. This process has been described in other papers in this issue and has lead to antigens Cb42 and Cb15 tested here.

A further complication arises with antigens like Cb48 which was identified purely on the basis of sequence similarity with the well characterised antigen PM48 from *L. cuprina* (SCHORDERET *et al.*, 1998). Since the native form of the Cb48 antigen in *C. bezziana* has not been identified, this antigen could only be evaluated as a recombinant protein.

This paper reports vaccination trials with four recombinant proteins, namely one each of Cb42 and Cb15 and two forms of Cb48. Evaluations were performed both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Recombinant antigens

The four antigens were recombinant proteins possessing a hexaHis tag as described (WIJFFELS *et al.*, 2000). HexaHis-Cb15 and hexaHis-Cb42 were both produced in *E. coli*. HexaHis-Cb48 was produced in the yeast, *Pichia pastoris* as well as in *E. coli*. The proteins were purified as described (PEARSON *et al.*, 2000).

Vaccination trials

Three vaccination trials were conducted in sheep which were: trial 1 vaccinating with hexaHis-Cb 15 and hexaHis-Cb42; and trials 2 and 3 vaccinating with hexaHis-Cb48. All sheep for trials were purchased from farms around Bogor. Sheep were screened for suitability by an ELISA, using an SDS extract of the *C. bezziana* peritrophic membrane as antigen. Some sheep showed significant antibody titres to the peritrophic membrane before vaccination. Although the reason for this is unclear, only sheep with low or negligible titres to the peritrophic membrane were used in vaccination trials.

For trial 1, a total of 18 male and female Javanese Thin Tail sheep (JTT) with ages between 1 – 3 years were weighed, then randomly allocated into 3 groups of 6 animals with comparable weight and age ranges. Group I animals were each vaccinated with 40 μg hexaHis-Cb15 in Montanide ISA-70 adjuvant (Seppic, Paris, France), Group II animals each received 50 μg hexaHis-Cb42 in Montanide ISA-70 and Group III PBS and Montanide ISA-70 as a control group. A Virtis blender was used for mixing the antigen and adjuvant with cooling on ice. HexaHis-Cb15 (260 μg in 750 μl) was added to 1.25 ml phosphate buffered saline (PBS). Procaine penicillin G (20,000 i.u.) and streptomycin (25 mg) was added in a 1 ml volume. Then 7.0 ml adjuvant was added (to maintain a ratio of 3 parts antigen solution and 7 parts adjuvant), and the blender speed was increased up to a medium speed for 2 min to emulsify. The adjuvant vaccine was divided into 6 doses (about 1.5 ml/animal). The adjuvant vaccine for hexaHis-Cb42 was prepared in the same manner as hexaHis-Cb15. Each dose contained 50 μg hexaHis-Cb42. For the control, six doses (about 1.5 ml/animal) were prepared from 3.0 ml PBS diluted into 7.0 ml adjuvant and emulsified as described above.

For the third trial, a total of 12 male and female JTT sheep with ages between 1 – 3 years were randomly allocated into 2 groups of 6 animals. Group I was vaccinated with hexaHis-Cb48 in Montanide ISA-70 using 1mg of recombinant antigen in 14 ml total volume, prepared as described above, vaccinating with a 2-ml dose.

All adjuvant vaccines were subcutaneously injected into the thigh area at a different site for every injection. The first booster injection was made 4 weeks after the first vaccination, and the second booster two weeks later. Challenges were 2 weeks after the second booster. Serum collection was done one day prior to each injection and at the time of larval recovery from *in vivo* challenge. The collected sera were kept in the freezer (-20°C) until used for ELISA and *in vitro* assays.

ELISA assays

Elisa assays against peritrophic membrane extract were performed as described (SUKARSIH *et al.*, 2000). For assays against *P. pastoris*-expressed Cb48, the antigen was used to coat ELISA plates at a concentration of 1μg/ml. The procedure was otherwise unchanged. Since a standard serum was not available, titres were calculated for an absorbance of 0.6, which was in the midpoint of the linear portion of a ELISA dilution series.

***In vivo* assay**

The procedure used was similar to that described for the measurement of growth and survival of *L. cuprina* larvae on sheep (EISEMANN *et al.*, 1989) and as described by PARTOUTOMO *et al.* (1998).

In vitro assay

The procedure used was similar to that described for *L. cuprina* (EISEMANN *et al.*, 1990) with adaptations for *C. bezziana* (SUKARSIH *et al.*, 2000). Briefly, medium containing Difco Noble agar, yeast extract and gentamycin sulphate was prepared to test the sera of 18 animals. After cooling the agar mixture slightly, the prewarmed serum (40°C) was added. The complete medium was dispensed as 5 × 1 ml aliquots into 20 ml plastic vials (5 replicates per serum), solidified, and the surface was scratched using clean forceps in every plastic vial. Ten healthy larvae were put onto pieces of approximately 3 mm thick plastic sponge (Wettex^{R}) and placed in a vial on the surface of medium so that the surface with the larvae was in contact with the medium. Vials were closed with fine gauze and incubated for 30 h at 37°C and 80% relative humidity. After this time, the numbers and weight of the surviving larvae was assessed. The procedure has been described in detail (SUKARSIH *et al.*, 2000).

Statistical analysis

The results of *in vitro* feeding assays were analysed using a student's T test of larval weights and larval recoveries from assays using individual sheep sera. Larval weights and larval recoveries themselves were normally the average of five replicate determinations with each serum. In cases where the assays were repeated the differences were examined by a two way ANOVA.

In vivo assays involved the infestation of four sites on each sheep. These four sites were repeated with each animal, namely right and left forequarter and right and left hindquarter. In case there was a systematic difference in the feeding performance of larvae in these sites, results of the assays were again analysed by a two-way ANOVA using treatment, whether vaccinated or control, and ring position as factors in the analysis. Analyses were performed using SigmaStat.

RESULTS AND DISCUSSION

The results of all vaccination trials with four recombinant antigens assessed *in vivo* and *in vitro* are listed in Table 1. Each vaccination group used five or six sheep with the same number of controls.

***In vitro* assays**

The results show that there was no effect in the *in vitro* assays on larvae feeding on any of the sera from sheep vaccinated with recombinant antigens, either in the recovery of larvae or in their mean weight. Recoveries were high, typically approximately 90%, and there was little variation in growth from serum to serum on either vaccinated or control sheep. This lack of response *in vitro* is strikingly different to the effects seen for larvae feeding on antisera to whole peritrophic membrane, where, in a series of experiments, the weight of feeding larvae was reduced by 73%, the recovery by 32%, and the overall mass of recovered larvae by 82% (SUKARSIH *et al.*, 2000). It is also different from the results of *in vitro* trials with antisera to individual peritrophins from *L. cuprina*. Feeding of *L. cuprina* larvae on antisera to *E. coli*-expressed Cb48 did produce a small, though statistically significant reduction in growth. The mean weight of control larvae in replicate experiments was 4.1 mg, while that for larvae feeding on antisera to Cb48 was 3.3 mg, a reduction of 19% ($P<0.05$). However, a trial of hexaHis-Cb15 conducted with merino sheep failed to show any effect when the sera were tested *in vitro* on larvae of *L. cuprina*.

Table 1. Mean weight and recovery of *C. bezziana* larvae from *in vitro* and *in vivo* assays on sheep vaccinated with hexaHis-Cb15 and hexaHis-Cb42 expressed in *E. coli* and hexaHis-Cb48 expressed in both *E. coli* and *P. pastoris*

Trial	Group	n	*In vivo*		*In vitro*	
			Larval no.	Larval wt.	Larval no.	Larval wt.
1	Cb15-*E.c.*	6	13.3	29.5	8.97 ± 0.82	3.22 ± 0.25
1	Cb42-*E.c.*	6	14.3	28.8	8.93 ± 0.48	3.24 ± 0.27
1	Controls	6	13.8	25.8	9.32 ± 0.58	3.20 ± 0.37
2	Cb48-*E.c.*	5			8.88 ± 0.34	2.29 ± 0.21
2	Controls	5			9.04 ± 0.68	2.33 ± 0.17
3	Cb48-*P.p.*	6	17.1	24.0	8.90 ± 0.35	2.59 ± 0.34
3	Controls	6	13.4	22.7	9.23 ± 0.34	2.82 ± 0.37

E.c. expressed in *E. coli*
P.p expressed in *P. pastoris*
Weights are expressed in mg

In vivo challenge

The results of *in vivo* assays on the same sheep are more striking. All of the vaccinated sheep showed a slight increase in the mean weight of recovered larvae and larval recoveries which were equal to or greater than those from control sheep in the same experiment. However, the effects were slight and only in one case, the mean recovery of larvae from sheep vaccinated with Cb48 expressed in *P. pastoris,* was the difference statistically significant ($F_{1,46}$ = 4.79, $p<0.05$). Larval weights and recoveries were independent of the site of infestation, that is, the position of the ring within which the controlled infestation took place.

Biology of the antigens

In principle, the lack of effect in the *in vitro* assays and the small effects *in vivo* could be due to an inability of antisera to the recombinant *C. bezziana* peritrophins to bind to the peritrophic membrane.

There is evidence that this is not the case. Firstly, all three antigens have been shown to be accessible in the larval peritrophic membrane to specific antibody. Immunofluorescence assays and immunogold labelled electron microscopy experiments with these antigens indicated their uniform distribution in the peritrophic membrane (EISEMANN *et al.,* 2000; VUOCOLO *et al.,* submitted; WIJFFELS *et al.,* in preparation). Immunological responses of sheep to the Cb48 antigen at least were also strong. The geometric mean antibody titre to *Pichia*-expressed Cb48 was 42000, measured as described in Materials and Methods, while the titre of a pool of antisera to whole peritrophic membrane used as an internal reference (SUKARSIH *et al.*, 2000) was only 5900. However, the antisera to Cb48 failed to give a significant titre against an SDS extract of peritrophic membrane. This may reflect the lack of solubility of native Cb48 in SDS extraction, or a low abundance.

Secondly, the ovine antisera to recombinant Cb48 from experiment 2 were also fed to *L. cuprina* larvae *in vitro*. This was done to examine the degree of immunological cross-reactivity between these two related antigens. Given that the degree of sequence identity predicted for the Cb48 and PM48 proteins is relatively low (~ 40%), strong immunological cross-reactivity might not be expected. Nevertheless, as described above, the effects of feeding on *L. cuprina* were statistically significant, though small.

The results presented here show that vaccination with some of the most abundant proteins of the peritrophic membrane cannot duplicate the effects seen *in vitro* or *in vivo* following vaccination with whole, native peritrophic membrane.

Immunisation of sheep with native and recombinant *L. cuprina* peritrophins leads to deleterious effects in *L. cuprina* larvae as measured by *in vitro* assays and *in vivo* challenge. The peritrophic membrane of the larvae is blocked after ingestion of specific antibody. This results in gelation or aggregation of material on the membrane, effectively blocking it and therefore starving the larvae. The lack of effects, particularly *in vitro*, on feeding *C. bezziana* larvae suggests that such blockage is not occurring in screwworm larvae, though this has not been established by direct observation. Physical parameters such as density of the peritrophins within and on the peritrophic membrane, the porosity of the membrane itself and protein and chitin fibril degradation and renewal are just some of the factors that potentially influence the ease with which the membrane may be affected.

CONCLUSIONS AND RECOMMENDATIONS

The data from the vaccine trials indicated that the three recombinant proteins, Cb15, Cb42 and Cb48, were not effective as vaccine antigens in sheep against infestation with *C. bezziana* larvae. This is in contrast to results with the whole native peritrophic membrane and in spite of the fact that antibody to the recombinant proteins can bind to the membrane *in vitro*. Comparison of larvae feeding on anti-recombinant peritrophin sera with those feeding on anti-whole peritrophic membrane is likely to give valuable information in understanding the nature of the protective response. Comparison of *C. bezziana* and *L. cuprina* larvae feeding on anti-peritrophic membrane sera may shed further light on the same question. A better understanding of the nature of effective vaccination would be crucial to the planning of future research directions.

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