

SUCCESSFUL VACCINATION AGAINST *BOOPHILUS MICROPLUS* AND *BABESIA BOVIS* USING RECOMBINANT ANTIGENS

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Current methods for the control of the cattle tick Boophilus microplus and the agent of bovine babesiosis, Babesia bovis are unsatisfactory. Effective immunological control of both parasites would have great advantages. However, naturally acquired immunity to the tick is generally unable to prevent serious production losses. A vaccine against the tick, based on a novel form of immunization, is being developed. A protective antigen has been isolated from the tick, characterized and produced as an effective, recombinant protein. A vaccine incorporating this antigen is currently undergoing field trials. In the Australian situation, improved tick control will probably increase endemic instability with respect to B. bovis. Fortunately, a trivalent, recombinant B. bovis vaccine has also been developed. This too is now undergoing pre-registration field trials.

Key words: vaccination – *Boophilus microplus* – *Babesia bovis* – recombinant antigens – field trials

The cattle tick *Boophilus microplus* and the haemoprotozoan parasite *Babesia bovis* remain serious problems for the cattle industry in tropical and sub-tropical Australia, as they are in other parts of the world. The economic cost of the tick in Australia alone has been estimated as more than \$ 100 million per annum (Cobon & Willadsen, 1990), largely as a result of the reduced growth rates of tick-infested cattle. The cost in South America is much greater (Horn, 1987). *B. bovis*, though not currently of such economic importance, is a continual threat to the cattle industry. In *Babesia* endemic areas, calves receive maternal antibody from immune mothers and are thus protected from *B. bovis* infections in early life. Mortality following infection is also greater in mature cattle than in young, immunologically naive ones. The greatest mortality from *B. bovis* infections therefore occurs where tick transmission of *B. bovis* is low and variable. However, in the Australian situation, endemic stability is reached only at levels of tick infestation (more than a hundred tick larvae attaching per day) where unacceptable losses of production occur due to the direct effect of the ticks on cattle. There is thus a continual conflict between the demands for increased control of one parasite and the need for endemic stability for the other.

Current control methods for both parasites are unsatisfactory. Tick control depends heavily

on the use of chemicals, with the accompanying problems of resistance to acarides and the possible presence of chemical residues (Nolan & Schnitzerling, 1986; Nolan et al., 1989; Schnitzerling et al., 1989). With *B. bovis*, a live attenuated vaccine has been in use in Australia for many years but this has number of disadvantages. Approximately 5% of animals react severely on vaccination and require drug treatment to survive. Since the attenuated vaccine is produced in splenectomized calves, contamination with other pathogens is always a possibility. Finally, reversion to virulence and to transmission by the vector can occur.

The development of effective immunological control of both parasites with non-living vaccines therefore has great economic importance to the Australian cattle industry. However, there are considerable technical difficulties in the production of vaccines against either parasite. Chief among these is the problem of identifying protective antigens. In the case of *B. microplus*, a further problems arises because of the nature of the host-parasite interaction. After extensive and prolonged exposure to the tick, cattle acquire a partial immunity through the development of an immediate sensitivity reaction to tick antigens. This immunity is usually unable to prevent serious production losses, particularly in *Bos taurus* cattle (Willadsen,

1980). A vaccine mimicking such "immunity" would seem to have little point. The first task in vaccine development was therefore to develop a better way of inducing protective immunity.

Development of a vaccine against B. microplus – It has been shown that vaccination of cattle with a crude extract of the internal organs of semi-engorged ticks can produce effective immunity (Johnston et al., 1986; Agbede & Kemp, 1986). This immunity acts by destroying the gut of the tick as it engorges (Kemp et al., 1986; Willadsen & Kemp, 1988), an effect which is not seen in ticks from a host with naturally-acquired immunity.

The first difficulty in developing a commercially useful vaccine from such observations was the need to identify the effective antigen or antigens. Considering the nature and complexity of the extracts used for vaccination, it was not expected there would be any observable correlation between antibody production and induced immunity. Antigens were therefore identified solely through a series of protein purification steps, the products of each step being analyzed by vaccination and challenge trials. One antigen capable of inducing immunity has been isolated, the Bm86 antigen (Willadsen et al., 1988, 1989). It is a membrane-bound glycoprotein, present in low abundance on the surface of the digest cells of the tick's gut. This protein represents less than 1 part in 10^5 of the material in a crude tick extract. Ingestion of antibody directed against this protein leads to lysis of the cells and gross damage to the gut of the tick through immunological mechanisms which are still not fully understood.

Production of a commercially useful vaccine requires that the antigen be produced cheaply and in large quantities. The only means for achieving this is by recombinant DNA technology. The gene coding for the antigen has been isolated and recombinant proteins produced in a variety of systems. Both the sequencing of the Bm86 gene and protein chemical studies carried out in parallel have revealed features of the antigen which would be difficult to duplicate in a commercially feasible expression system. The antigen is heavily glycosylated, carrying approximately 24 kD of carbohydrate with four potential N-linked glycosylation sites. It is membrane-bound via a glycosyl phosphatidylinositol membrane anchor (Richardson et al., submitted for publication) and, most importantly, it contains 10 mole% cysteine residues. Not surprisingly, expression of the antigen gene as a β -galactosidase fusion protein in *E. coli* resulted in the formation of insoluble inclusion bodies. This product was nevertheless effective as a vaccine antigen, though less so than the native antigen (Rand et al., 1989) (Table I).

Two approaches have been adopted to improve the antigenicity of the recombinant product. The first is solubilization of the antigen under reducing conditions followed by controlled re-oxidation. The second is expression in a baculovirus vector in S.f.9 cells. The products of both systems induce immunity to ticks that is similar to that induced by the native antigen. Following vaccination, the number of ticks maturing on vaccinated cattle is reduced as is their engorgement weight and their egg laying capacity. The overall effect on a tick population in one life cycle can be measured by a combination of these three effects. If the

TABLE I

Efficacy of Bm86 native antigen and recombinant proteins

Antigen	% reduction relative to control infestations			
	Tick number	Mean wt.	Egg ratio	Reproductive capacity
Native Bm86, 7 μ g	70	29	53	91
Native Bm86, 50 μ g	61	37	70	93
<i>E. coli</i> , BTA 1752	24	31	56	77
<i>E. coli</i> , BTA 1696	27	33	78	89
<i>Baculovirus</i> , BTA 2080	45	30	77	91

Cattle were infested with 1000 *B. microplus* larvae per day and the adult female ticks engorging three weeks later collected, counted, weighed and their egg laying capacity assessed. The egg ratio is the weight of eggs laid by one gram of adult female ticks. The reduction in reproductive capacity is a combination of the reduction in numbers of engorging ticks, their weight and their egg laying capacity.

overall effect on the reproductive capacity of the ticks is used as a measure, both the refolded *E. coli*-expressed antigen and the baculovirus antigen are as effective as the native antigen, since all reduce reproductive capacity by greater than 90% in one cycle (Table I). Since it is very unlikely that the *E. coli* product is refolded completely in the correct conformation, this has established that neither a perfectly native structure nor presentation in a membrane-anchored form nor correct glycosylation is needed for protective antigenicity. With respect to the last point, it has been demonstrated recently that the Bm86 carbohydrate is not protective (Willadsen & McKenna, 1991).

In a field situation, the expected effect of the vaccine would not be principally in direct killing of ticks within a single generation but rather in progressive control of tick numbers in successive generations through a reduction in reproductive capacity. Field trials in central and south-eastern Queensland have confirmed this expectation.

The effects of vaccination appear to be specific for the adult stage of the life cycle, probably because this corresponds to the period of maximum uptake of blood. The effects are greatest for the events of the late adult state – engorgement weight and egg laying – for the same reason. This has important consequences for the transmission of *Babesia*.

Impact of enhanced tick control on the endemic stability of B. bovis – A stable epidemiological situation for babesiosis is governed by the inoculation rate which is sufficient to infect all calves by six to nine months of age. Resistance to the disease up to this age is mainly due to the persistence in the calves' blood of colostral antibodies which have been obtained from immune mothers. Following infection, immunity persists for a minimum of four years (Mahoney et al., 1973). The minimum daily inoculation rate for stability of babesiosis is 0.01. This rate will effectively ensure infection of all animals in a herd within nine months of birth. From field estimates of the rates of tick infection with *B. bovis*, this equates to a daily tick infestation rate of 100 larvae per day per cow for 270 days (Mahoney et al., 1981).

With high levels of tick control, the tick infestation rate and hence the *B. bovis* inoculation rate is likely to fall well below this level,

rendering the population susceptible to fatal attacks of *B. bovis*. It is for this reason that any control program for ticks must concurrently use immunological control of *B. bovis*. Simultaneous application of immunological control for both *B. microplus* and *B. bovis* is clearly highly desirable.

Development of a vaccine against B. bovis – Strong protective immunity against *B. bovis* was first demonstrated by Mahoney (1967) who showed that killed *Babesia*, when mixed with adjuvant, protected cattle against subsequent virulent challenge with that organism. This work was extended by the group at CSIRO who undertook to identify the protective antigens through a series of chromatographic and electrophoretic separation procedures. After each series of separations, fractions were tested for protective activity by vaccination and parasite challenge. A total of about 80 vaccination trials was carried out over a five year period, leading to the development of sequential purification procedures of a number of protective, antigenic fractions. These antigen fractions were then further resolved using collections of monoclonal antibodies to the components of the fractions. This procedure has led to the successful identification of a number of protective antigens, three of which have now been produced in quantity by recombinant DNA technology.

It is important that in this pragmatic approach, no correlation was assumed between any immunological parameter and protection against the parasite. In this, the *B. bovis* vaccine development has paralleled the development of the *B. microplus* vaccine. The approach contrasts however with that of isolating antigens recognized by sera from immune hosts, a procedure in common use.

Western blotting of *B. bovis* material with sera from cattle with naturally acquired immunity to *B. bovis* shows a complex series of antigens, a number of which consistently appear to be immunodominant. Four of these have been tested either as native proteins or as recombinants, but have failed to show any protective effect. Furthermore, two protective recombinant antigens contain tandem repeat segments which, if used alone, are non-protective or even immunosuppressive, although they themselves stimulate antibody production effectively. The protective antigens identified by repetitive cycles of separation, vaccination and

parasite challenge are present only in small amounts. Screening an expression library with immune serum then will almost invariably select immunodominant but non-protective antigens.

Antigen 12D3 – The ability of an antigen designated 15B1, isolated by affinity chromatography with an IgM class monoclonal antibody, to provide partial protection against *B. bovis* has been described previously (Wright et al., 1983). This antigen was subsequently shown to be immunologically cross-reactive with a second monoclonal antibody of IgG₁ class, designated 12D3. This second antibody facilitated the isolation, testing and N-terminal sequencing of the antigen. Vaccination with the native antigen leads to a ten-fold reduction in peak parasitemia on challenge with homologous, virulent *B. bovis*, with increased survival of the host.

The gene coding for the 12D3 antigen has been isolated and fully sequenced. The gene product is a 38kD protein with 10% cysteine residues, two epidermal growth factor-like domains (Doolittle et al., 1984; Rees et al., 1988) and a signal sequence consistent with transfer across the parasite membrane. Immunofluorescence has located the antigen within the parasite cytoplasm and in the cytoplasm of the infected erythrocyte. Not surprisingly, considering the high cysteine content, expression in *E. coli* as either a β -galactosidase or GST-fusion protein produced insoluble inclusion bodies. However, solubilization under denaturing and reducing conditions followed by re-oxidation gave a product that was protective on vaccination.

Antigen 11C5 – The antigen was isolated using one of a battery of monoclonal antibodies raised against a fraction "b" which had been

shown to be protective (Goodger et al., 1985). Screening of a λ gt11 expression library with the monoclonal antibody W11C5 led to the isolation of 280 kD β -galactosidase fusion protein. Vaccination of cattle with this material either as a crude *E. coli* lysate or as an SDS PAGE purified band conferred good protection. This antigen was later expressed as a glutathione S-transferase fusion protein in the pGEX expression plasmid.

Interestingly, the DNA sequence contains a 5' region with 19 copies of a 90 bp repeat sequence and a 3' non-repeat region. Both regions have been cloned and expressed separately. The repeat region was immunogenic but not protective, while the non-repeat region was protective.

Proteinase antigen 21B4 – It has previously been shown that partially purified proteinase preparations from *B. bovis* are able to induce protective immunity in cattle (Commins et al., 1985). One of the proteins from this fraction has been cloned using the monoclonal antibody T21B4 and is now available as a GST fusion. A small fragment of 51 amino acids has been shown to be protective (R.E. Casu, unpublished; Gale et al., 1991).

Synergism between recombinant antigens; demonstration in field trials – In an initial field trial using a natural challenge of cattle with *Babesia* infected ticks, 12.5% of cattle vaccinated with the 11C5 recombinant antigen required treatment, compared with 40% of control cattle. In the same trial, a second group were vaccinated with both the 11C5 and 12D3 recombinant proteins. Only 10% of this group required treatment. It was decided to further investigate the possibility of synergistic effects with a field trial of a trivalent vaccine incorporating the 12D3, 11C5 and 21B4 recombinant

TABLE II

Field trial of a recombinant *Babesia bovis* vaccine

Group	No. cattle	Mean max. parasitemia / μ l	Treatment rate
Non-vacc. control	19	6960 \pm 150	13/19
QDPI Vacc. A.	40	1206 \pm 40	2/40
B.	40	141 \pm 27	1/38
Recombinant vacc.	18	824 \pm 35	0/18

The QDPI vaccinated group were vaccinated with the commercial attenuated vaccine. Part A of this group refers to the parasitemia and morbidity resulting from vaccination with live attenuated parasites. Part B refers to the results of subsequent challenge with *B. bovis* in the field.

antigens. Over a period of three months, cattle were subjected to heavy challenge with *B. bovis*-infected ticks, coincidental with a natural *B. bigemina* challenge. The results are listed in Table II. The trivalent recombinant antigen vaccine provided complete protection against mortality as well as out-performing the currently available attenuated live vaccine. In this trial, 67% of non-vaccinated controls and 5% of animals receiving the live attenuated vaccine required treatment. Although this result must be confirmed under more extended field conditions, it does establish the feasibility of vaccinating against babesiosis with a commercially viable, defined antigen vaccine.

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