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IMMUNOLOGIC CONTROL OF A PARASITIC ARTHROPOD

Identification of a Protective Antigen from *Boophilus microplus*[1]

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Cattle can be vaccinated against the tick *Boophilus microplus* by inducing an immunologic reaction against Ag in the tick gut. The uptake of antibody during feeding leads to severe damage to the parasite. One of the responsible tick gut Ag has now been purified and characterized: the Bm86 Ag. It is a membrane-bound glycoprotein present in very low abundance in extracts of partially engorged adult female ticks. It has an apparent m.w. of 89,000, an isoelectric point of 5.1 to 5.6 and an affinity for wheat germ lectin. Microgram amounts of this Ag are able to induce effective protection in cattle against the parasite, as shown by the decreased survival of ticks on vaccinated cattle and a reduction in engorgement weights and egg laying capacity of the survivors. Antisera to the Ag react with the surface of digest cells in the tick gut. As a result of the reaction with antibody, the endocytotic activity of these cells, which is a critical step in bloodmeal digestion in this tick, is strongly and rapidly inhibited. A number of peptides from this Ag, produced by digestion of the reduced and alkylated protein with endoproteinase lys-C, have been sequenced. One peptide has significant amino acid sequence homology with the epidermal growth factor precursor and a second peptide has homology with a putative protective antigen from *Plasmodium falciparum*.

Blood feeding ectoparasites are a major problem to both human and veterinary health world wide, both as debilitating agents themselves and as vectors of disease. Current control is largely limited to the use of chemicals, with the attendant problems of the development of resistance by the parasite to the chemicals and the persistence of chemical residues. Immunologic methods of control would offer many advantages, but no successful vaccine has yet been developed against any ectoparasite. In part, this is due to the nature of the host-parasite interaction. It is frequently found that even after extensive exposure of a host to an ectoparasite, the immunity that is acquired by a host, if any, is still inadequate to prevent deleterious effects to the host.

Although there is no general solution to the problem of inadequate naturally acquired immunity, one approach that may have relevance to many blood-feeding ectoparasites has been demonstrated in the control of the tick *Boophilus microplus*. This is an economically important parasite of cattle throughout many tropical and subtropical countries, including Australia and Central and South America. Cattle acquire a partial immunity to the parasite after extensive natural exposure, due largely to the acquisition of an immediate hypersensitivity reaction to the ticks (1) which is, nevertheless, unable to prevent serious losses to cattle production. However, if cattle are vaccinated with Ag from the tick gut, then subsequent ingestion with the blood meal of bovine antibodies directed against the gut leads to gross damage to the tick (2, 3). The effects range from a reduction in engorgement weight of the ticks and a reduction in egg laying to death of the parasites (4). This is an immunity quite different from that acquired naturally, and it is one we have referred to as an immunity to "concealed Ag" (4).

Here, we describe the identification of an Ag that elicits an immunologic response in cattle capable of protecting them against ticks, as well as initial studies on the localization of the Ag and the mode of action of the immunologic response.

MATERIALS AND METHODS

Materials

The culture of *B. microplus* and the isolation of membrane material have been described elsewhere (5). Wheat germ lectin was isolated (6) and coupled to CNBr-Sepharose as described by the manufacturer (7) at a concentration of approximately 8 mg/ml Sepharose. Zwittergent 3-14 was obtained from Calbiochem (Sydney, Australia) and endoproteinase lys-C from Boehringer Mannheim (Sydney, Australia). Fluorescein-labeled rabbit antibovine Ig was a gift from R. Pearson and fluorescein-labeled sheep antirabbit Ig was obtained from Wellcome. Recombinant Ag used in the production of rabbit antisera were a gift from Biotechnology Australia.

Isolation of B. microplus Ag, Bm86

Step 1: preparation of membrane extracts. A crude preparation of membrane and particulate material was obtained from 988 g of ticks, representing approximately 50,000 hand-picked ticks. The material was isolated as three fractions sedimenting at 600, 20,000, and 100,000 × *g* as a matter of convenience and stored at −20°C until required. The three fractions were thawed and pooled for Ag

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extraction. A total of 31,200 mg of protein was diluted to 5 mg/ml in 0.05 M Tris acetate buffer pH 7.6 and extracted with 10 mg/ml Brij 35 at 37°C for 1 h. The extract was centrifuged at 10,000 × *g* for 30 min, the supernatant discarded and the precipitate, 17,200 mg protein, suspended to 5 mg/ml protein and extracted with 10 mg/ml Zwittergent 3–14 for 90 min at 37°C and then centrifuged as before.

Step 2: lectin affinity chromatography. The supernatant was mixed with 130 ml wheat germ lectin-Sepharose and stirred overnight at 20°C. The affinity support was allowed to settle, decanted, poured into a column 2.5 × 26 cm then washed with 0.05 M Tris acetate buffer containing 1% Zwittergent 3–14 and bound material eluted in the same buffer to which had been added 100 mg/ml N-acetyl glucosamine. This absorption and elution procedure was repeated in the same way with the supernatant of the first absorption and the two fractions of lectin-binding material pooled. This material, 160 mg, was then chromatographed on a 1.5 × 12 cm column of Con A-Sepharose in 0.05 M Tris chloride buffer pH 7.5 containing 0.1 M ammonium thiocyanate and 1% Zwittergent. Bound protein was eluted with 50 mg/ml methyl-α-D-mannopyranoside.

Step 3: preparative IEF. The material that had bound to Con A-Sepharose was subjected to IEF using Pharmalyte 4–6.5 and IEF Sephadex in 1% Zwittergent according to the manufacturer's instructions, for a total of 14,000 Vhr, at 10°C. At this stage, individual fractions from the preparative IEF were analyzed by SDS PAGE and those containing the required protein pooled. The pI range pooled was 5.1 to 5.6.

Step 4: HPLC gel filtration. To remove ampholines and exchange the detergent from Zwittergent to SDS, pooled IEF fractions were bound to a small column of wheat germ lectin-Sepharose, washed with 0.05 M HEPES buffer pH 7.0 containing 0.1% SDS, then eluted with 100 mg/ml N-acetyl glucosamine in this buffer. The HPLC system used, in series, an Si200 Polyol guard column, TSKgel G4000 SW and G3000 SW columns, each 0.75 × 30 cm, with an eluant of 0.05 M HEPES buffer, 0.1 M sodium thiocyanate, 0.1% SDS pH 7.0. Fractions containing the required protein were pooled after analytical SDS gel electrophoresis, concentrated, and the chromatography repeated to obtain better purity of the final product. The yield of Ag was 185 μg.

Vaccination of Cattle and Parasite Challenge

Cattle used were *Bos taurus* breed (Hereford) approximately 12 mo old, previously unexposed to *B. microplus*. Two vaccination trials were carried out. In trial 1, three cattle were given three vaccinations each of 2.3 μg of protein, 4 wk apart, with the first two vaccinations in CFA, the third without adjuvant. In trial 2, four cattle each received three injections of 17 μg of Ag, the first and third injections in CFA, the second in IFA. In each trial, the same number of control cattle received adjuvant alone. The culture of *B. microplus* larvae and challenge of cattle with parasites have been described previously (5). Briefly, cattle were infested individually with 1000 larvae per day for 3 wk. After about 21 days, engorged, adult female ticks dropped from the cattle and, each day, were collected, counted, weighed, examined for obvious signs of damage and assessed for egg laying capacity.

Peptide Sequence Analysis

Aliquots of approximately 40 μg of Ag were reduced and alkylated (8) before digestion with endoproteinase lys-C in 0.10 M Tris chloride buffer pH 8.2 containing 4 M urea. Digestion was started by adding 0.024 U of enzyme to the Ag at 37°C, to be followed by a further 0.024 U of enzyme after 4 h. The hydrolysis was allowed to proceed for 24 h. Peptides were purified by reverse phase HPLC on Aquapore RP-300 C-8 columns using gradients of acetonitrile in aqueous 0.1% heptafluorobutyric or TFA. The peptides were then sequenced with an Applied Biosystems sequencer (Foster City, CA).

Indirect Fluorescent Antibody Labeling of Tick Gut Cells

Semiengorged adult female ticks, 4 to 6 mm in length, were dissected under cold insect Ringer's solution, which was based on the physiologic saline used by Prince and Berridge (9) but with the addition of 0.1% gelatin and 100 U/ml streptomycin and penicillin. All subsequent washing steps were in the same, cold Ringers. The gut was everted with a fine steel needle, washed, reacted with bovine or rabbit antiserum for 2 h, washed, then reacted for 2 h with a 1/50 dilution of either fluorescein-labeled rabbit antibovine Ig or fluorescein-labeled sheep antirabbit Ig as appropriate. After washing, the tissues were examined under a Zeiss fluorescence microscope.

Isolation of Tick Gut Cells and Observation of Endocytosis

Tick guts, dissected as above, were split longitudinally and washed three times in cold insect Ringer's solution. This removed gut contents and any poorly attached gut cells. The large, lumen side gut cells (digest cells) were removed from the basement membrane by gently fleecing the cells with the leading edge of a flat probe, the suspension filtered through gauze and the cells washed three times with cold Ringer's solution.

The effect of antibody on endocytosis was observed by mixing 50 μl cell suspension, containing approximately 2.5×10^5 cells/ml with 50 μl of antiserum diluted in insect Ringer's solution and incubating for 2 h at 30°C. If desired, C was inactivated by heating serum at 56°C for 30 min before incubation with cells. Fluorescein-labeled BSA with a molar ratio of fluorescein:protein of 2.1 to 2.6 was added to a final concentration of 3 mg/ml. After 30 min at 30°C, the cells were washed in cold Ringer's, resuspended to 50 μl and examined under a Zeiss fluorescence microscope.

Other Methods

Methods for electrophoresis and the estimation of protein concentration were as described previously (5) except that the concentration of purified antigen was estimated from the absorbance at 280 nm, assuming a specific absorption of 1 $(g/liter)^{-1}cm^{-1}$.

RESULTS

Purification of the Ag Bm86. The partial purification of a protective antigenic fraction has already been described (5). A tentative identification of the actual Ag in this still complex mixture was made after preparation of the Ag as described previously (5), followed by affinity chromatography on wheat germ lectin-Sepharose and then preparative SDS gel electrophoresis. Vaccination of cattle with electroeluted fractions suggested that a glycoprotein with a m.w. of approximately 89,000 was the effective Ag. This conclusion was only tentative for a number of reasons: the Ag still contained significant amounts of contaminants, the amount of protein injected was low and the effects on ticks were small (P. Willadsen, G. A. Riding, and R. V. McKenna, unpublished observations).

Another purification procedure was therefore designed to isolate this protein in larger amounts and satisfactory purity, using the known physical properties of the putative protective Ag. These characteristics included an apparent m.w. of 89,000 and known mobility on SDS PAGE, an isoelectric point of 5.1 to 5.6 on IEF in detergent (5) and an affinity for both wheat germ lectin and Con A. This was sufficient information for the protein to be isolated by the procedure described in *Materials and Methods* and shown schematically in Figure 1 for a typical preparation. Figure 1 also shows SDS-PAGE of the two Ag preparations used in vaccination trials. Four consecutive preparations of the Ag gave yields of 24 to 187 μg/kg ticks, with a mean of 106 μg/kg.

Vaccination of cattle and parasite challenge. Cattle were vaccinated with purified Ag in two experiments as described in *Materials and Methods*, then challenged with ticks 2 wk after the third vaccination. This involved infesting the cattle individually with 1000 larvae per day for 3 wk. Engorged, adult, female ticks were collected on completion of the parasitic part of the life cycle approximately 3 wk after each day's infestation. The effects of vaccination on the adult female ticks were assessed by a number of parameters: the number and average weight of ticks maturing each day on individual cattle and a visual estimate of the percentage of ticks in each sample that were damaged (5). Samples of engorged female ticks

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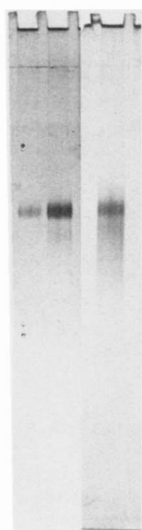

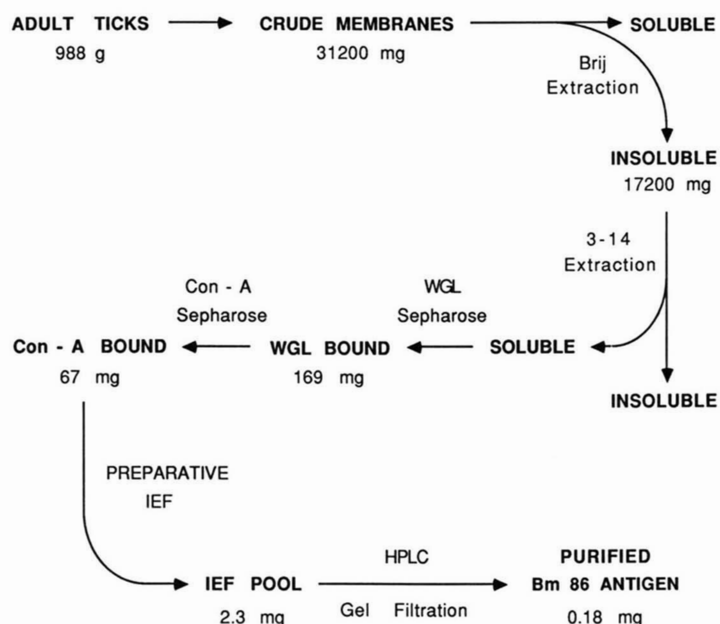

Figure 1. Purification of Bm86. The inset shows 0.2 and 0.7 μg of Bm86 used in trial 2 and 1 μg of Bm86 for trial 1.

TABLE I
Vaccination of cattle with purified Boophilus microplus Ag[a]

Animal	Group	No. of Ticks/Day	Tick Weight (mg)	% Damage	Weight Eggs / Weight ticks
Trial 1					
26	Control	199	224	6	0.49
29	Control	237	231	3	0.52
31	Control	227	220	1	0.47
30	Vaccinate	25	152	86	0.16
32	Vaccinate	135	175	79	0.25
34	Vaccinate	38	152	70	0.22
Trial 2					
114	Control	197	230	1	0.56
115	Control	254	247	2	0.56
120	Control	224	220	2	0.57
123	Control	264	267	2	0.58
119	Vaccinate	55	143	90	0.18
122	Vaccinate	118	155	92	0.16
124	Vaccinate	89	157	91	0.19
125	Vaccinate	100	156	92	0.16

[a] In trial 1, the results for no. of ticks per day, weight, and estimates of damage are the means of 17 daily estimates. Egg laying figures are the means of nine estimates. For trial 2, the results for tick no., weight, and damage are the means of 15 days' estimates and those for egg laying of 8 days' estimates. The SE of the data are not shown individually. However, the largest SE for the parameters recorded were: for the mean daily tick count for any control animal, 23 ticks; for the mean daily tick count on any vaccinate, 14 ticks; for daily tick weight, 10 mg; for the egg laying ratio, 0.04 and for the estimates of damaged ticks on vaccinated cattle, 5%.

```
 1. (K)DPDPGK
 2. (K)WYEDRVLEAIRTSIGK
 3. (K)LQACEHPIGEWCMMYPK
 4. (K)EAGFVCK
 5. (K)GPDGQCINXXK
 6. (K)AGVSTNENEQLEQADK
 7. (K)DQEAAY
 8. (K)CPRDNMYFNAAEK
 9. (K)ANCQCPPDTRPGEIGCIE
10. (K)ESSICXDFGNEFCRNAECEVVP
11. (K)TRECSYGRCVESNPSK
12. (K)AYECTCPRAFTVAEDGIXCK
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Figure 2. Amino acid sequences for Bm86 peptides. The initial K is assumed to be present because of the specificity of endoproteinase lys-C. Residues that could not be assigned with confidence are indicated as X. The one letter code for amino acids has been used.

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188
 E S S I C T A F S A Y N I L N   Plasmodium falciparum antigen
 : : : : :     : .         :
 E S S I C X D F G N E F C R N A E C E V V P   Peptide 10


383
 S Y H C T C P T G F V L L P D G K Q C H      EGF-P
 . :   : : : :   . :   .     : :     : .
 A Y E C T C P R A F T V A E D G I T C K      Peptide 12
 .     :   : :     :     . .   : :   : :
 G P R C I C P A G S V L G R D G K T C T      EGF-P
424
```

Figure 3. Sequence homologies between Bm86 peptides and epidermal growth factor precursor (*EGF-P*) and *Plasmodium falciparum* Ag showing identical (:) and homologous (.) residues.

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were also incubated and the weight of eggs laid per gram of engorged ticks determined (5); the results are shown in Table I.

Overall, in the two trials, the number of ticks surviving to engorgement was reduced by 65%, the average weight of those ticks which managed to engorge was reduced by 33% whereas an average of 86% of the surviving ticks showed visible damage. Not surprisingly therefore, the egg laying capacity of surviving female ticks, as measured by the conversion of the weight of engorged female ticks into eggs, was reduced from an average of 54% for the seven control cattle to 19% for the seven vaccinates. Overall, the efficacy of vaccination may be gauged from the reduction in the number of larval progeny from one complete parasitic life cycle. This is the product of the effects on tick numbers, weights, and fecundity and, for the two trials, gave a 92% reduction in larval numbers. The result is particularly impressive in view of the small amount of Ag injected (Table I).

Amino acid sequence and structural features of peptides from Bm86. The Ag was reduced, alkylated, digested with endoproteinase lys-C, peptides purified by HPLC and a total of 12 peptide sequences obtained by gas phase microsequencing. The sequences of these peptides are listed in Figure 2. Using oligonucleotide probes designed from this sequence information, the gene coding for the Ag has been successfully isolated (K. N. Rand, T. Moore et al., manuscript in preparation). The structure of the Ag will be discussed in more detail in a subsequent report, but peptides 10 and 12 have interesting amino acid sequence homologies with previously characterized proteins, as shown in Figure 3. Peptide 10 has a short but significant homology with a putative protective antigen from *Plasmodium falciparum* (10) whereas peptide 12 has a more extended and highly significant sequence homology with two sections of the epidermal growth factor precursor molecule. A second striking feature of the peptide sequences is the high proportion of cysteine or half-cystine residues. Of the 182 amino acids sequenced, which must represent at least 20% of the molecule, 10.4% are cysteines.

Localization of Ag. Indirect immunofluorescence using either bovine antisera to the native tick Ag Bm86 or rabbit antisera to a recombinant protein produced in *Escherichia coli* shows that the Ag is located on the surface of tick gut digest cells (Fig. 4). Prevaccination sera or sera from animals injected with adjuvant alone gave no reaction at the same dilution (Fig. 4). More detailed localization of the Ag in fixed sections has proved to be relatively difficult, due perhaps to the low abundance of the Ag.

Effects of vaccination sera on digest cells. Suspensions of digest cells were incubated with antiserum and then their ability to endocytose fluorescein-labeled BSA was observed. After preincubation of the cells with bovine antiserum to the Bm86 Ag from animal 30 at a dilution of up to 1/50, endocytosis by the digest cells was very strongly inhibited. At antiserum dilutions of 1/10

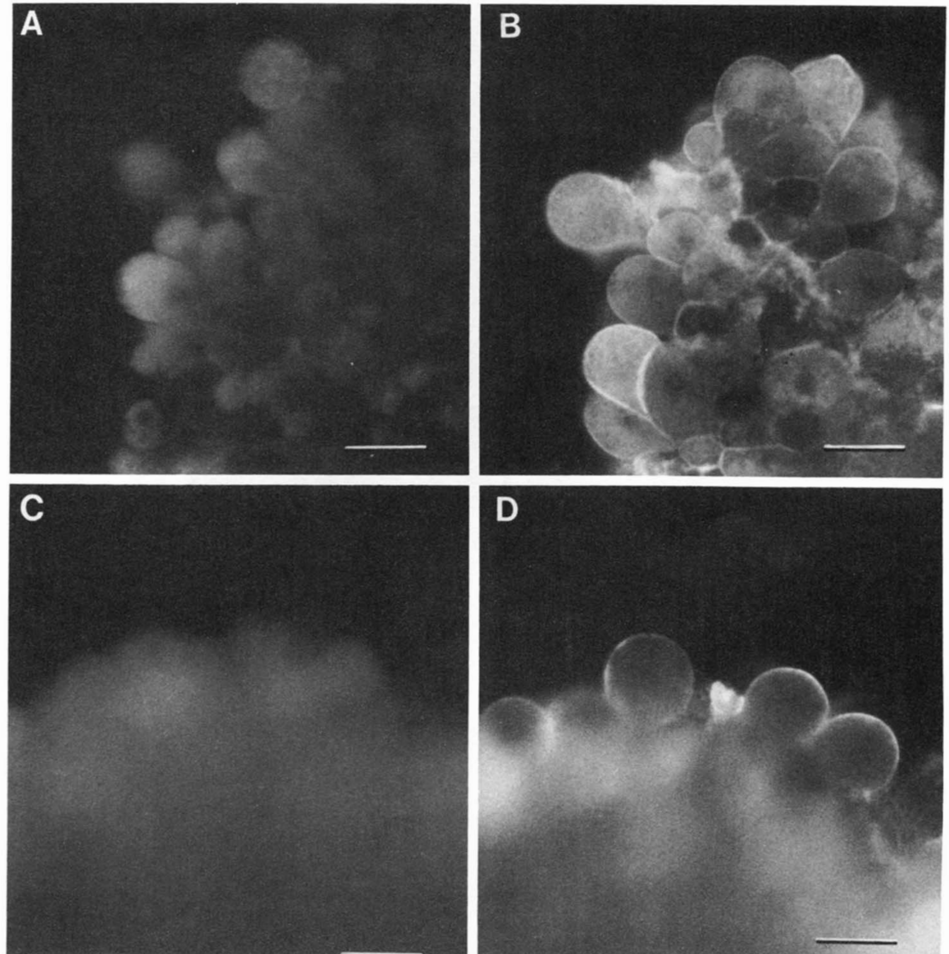

Figure 4. Immunofluorescence of tick gut cells. *A*, Cells incubated with normal rabbit serum, diluted 1/50 (*B*) cells with rabbit antiserum against recombinant Bm86 Ag, diluted 1/50 (*C*) cells with bovine serum no. 34, before vaccination, diluted 1/100 (*D*) cells with bovine antiserum no. 34 to native Bm86, diluted 1/100. The scale bar is 40 μm.

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no endocytosis at all was visible. Other bovine antisera at the same dilution produced complete or strong inhibition of endocytosis (Fig. 5). Heat inactivated antisera appeared qualitatively to be as inhibitory as unheated sera. Prevaccination serum or serum from control cattle had no effect at this dilution (Fig. 5).

DISCUSSION

The suggestion that blood feeding parasites may be damaged by an immunological reaction against their internal organs is not new (11, 12) but the work with *B. microplus* is the only detailed study of this effect (2–4). However, turning this into a practical vaccine demands the solution of a number of problems. One of the most difficult is the identification of Ag which, taken in isolation, work well enough for a commercial vaccine to be feasible. The difficulty is increased when the starting material for Ag isolation is a crude homogenate of a whole parasite (2) and the parasite is, moreover, so host specific that all Ag trials must be done by vaccinating cattle. That these problems are not insurmountable is shown by the successful identification of the Ag described in this report.

The Ag appears to be a minor cell membrane component. The amount of Ag isolated from crude membrane preparations is small. Although this could be due to a low yield on purification, the isolation was routinely monitored by SDS-PAGE and, after the first affinity steps, no major losses of Ag could be seen. The apparent low abundance of the gene in cDNA libraries (K. N. Rand, T. Moore et al., manuscript in preparation) is also indicative of a protein of low abundance.

It would be fascinating to know the function of this protein, but the only evidence is indirect. The homology with the epidermal growth factor precursor is interesting, because this molecule contains a repeat structure around a six cysteine unit, the epidermal growth factor repeat, which has been found in a total of 27 different proteins. Part of such a structure is in the region homologous to Bm86. All these proteins are extracellular or cell surface bound and many are growth factor-like molecules or are

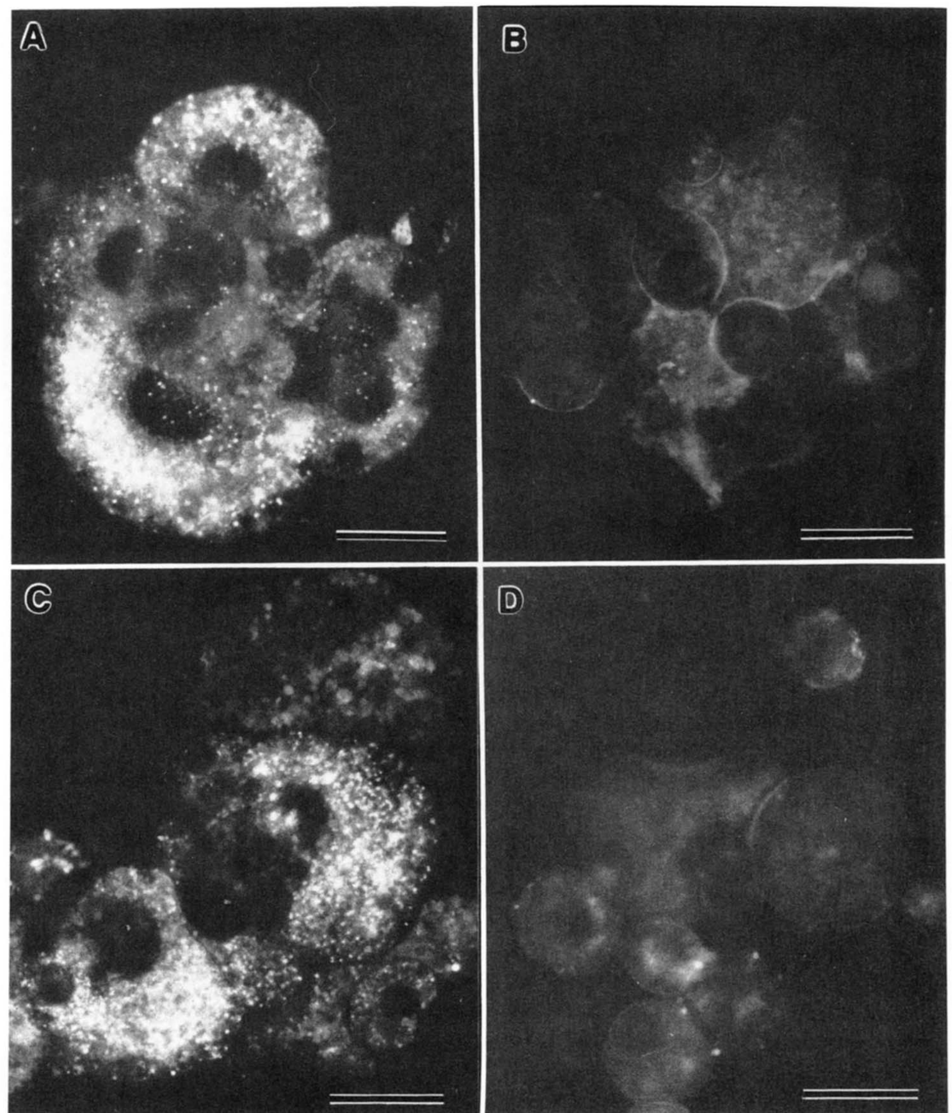

Figure 5. Endocytosis by tick gut cells. Cells were incubated with 1/10 dilution of bovine serum, then fluorescein-labeled BSA added. The sera were: (*A*) control no. 26 (*B*) vaccinate no. 30 (*C*) control no. 114 (*D*) vaccinate no. 122. The scale bar is 40 μm.

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involved in blood coagulation. The *Plasmodium* Ag which shows another sequence homology with the Bm86 Ag is one that also contains these EGF-like domains (10). The apparent high cysteine content of the antigen is also characteristic of many extracellular proteins, especially cell surface receptors (13, 14). The full sequence provides further information about such analogies.

The histologic examination of ticks engorging on cattle vaccinated with crude and partially purified Ag has shown consistently that there is destruction of the digest cells (3, 4). These are the cells that are specialized for the endocytosis and digestion of the tick's blood meal. It is consistent with this that antisera to the purified Ag and also to a recombinant form of the Ag react with the surface of these cells. Inasmuch as the recombinant protein was produced in *E. coli*, reaction between rabbit antisera to the recombinant and tick gut cells will be due to protein determinants rather than to common carbohydrate determinants. It has been shown previously in several ways that bovine antisera from successfully vaccinated cattle rapidly inhibit the normal functioning of the digest cells of the tick gut (4, 15). It has now been demonstrated that antisera reacting against Bm86 inhibit the endocytotic activity of isolated digest cells and, furthermore, that this inhibition is not C dependent. Whether this is the lethal lesion in the ticks is still unknown.

This work demonstrates the possibility of vaccinating against ticks using defined Ag to produce an immunologic mechanism which does not occur in the natural host-parasite interaction. There is every reason to expect that success will be achieved with other tick species and, perhaps, with other hematophagous ectoparasites (16). For example, there is recent evidence that both the human body louse (17) and the mosquito *Aedes aegypti* (18) may be damaged by immunologic mechanisms analogous to the one we have described for the tick.

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