

Antibody-mediated inhibition of the growth of larvae from an insect causing cutaneous myiasis in a mammalian host

(peritrophic membrane/*Lucilia cuprina*/immunological control)

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ABSTRACT Many insects feed on blood or tissue from mammalian hosts. One potential strategy for the control of these insects is to vaccinate the host with antigens derived from the insect. The larvae of the fly *Lucilia cuprina* feed on ovine tissue and tissue fluids causing a cutaneous myiasis associated with considerable host morbidity and mortality. A candidate vaccine antigen, peritrophin 95, was purified from the peritrophic membrane, which lines the gut of these larvae. Serum from sheep vaccinated with peritrophin 95 inhibited growth of first-instar *L. cuprina* larvae that fed on this serum. Growth inhibition was probably caused by antibody-mediated blockage of the normally semipermeable peritrophic membrane and the subsequent development of an impervious layer of undefined composition on the gut lumen side of the peritrophic membrane that restricted access of nutrients to the larvae. The amino acid sequence of peritrophin 95 was determined by cloning the DNA complementary to its mRNA. The deduced amino acid sequence codes for a secreted protein containing a distinct Cys-rich domain of 317 amino acids followed by a mucin-like domain of 139 amino acids. The Cys-rich domain may be involved in binding chitin. This report describes a novel immunological strategy for the potential control of *L. cuprina* larvae that may have general application to the control of other insect pests.

Many insects feed on blood, tissue, or tissue fluids of mammals. The insect feeding process itself is often detrimental to the host and in addition many of these insects are vectors for the transmission of viral, protozoal, and helminth parasites that cause considerable host morbidity and mortality, particularly in humans and livestock animals. Current strategies for control of insect pests in livestock industries are becoming inadequate because of rapid and widespread development of insecticide resistance and concerns relating to the presence of chemical residues derived from these insecticides in livestock products and the immediate environment. Thus, alternative means of controlling these insects are required.

One possible approach is to vaccinate the host against the offending insect life stage by using an insect gut antigen (1, 2). The gut of most insects is lined with a peritrophic membrane (PM or peritrophic matrix) at least at some stage in the life cycle (3). This semipermeable membrane is composed of chitin, proteoglycans, and proteins. The functions of the PM involve facilitating the digestive process and protecting the gut epithelial cells from invasion by microorganisms and parasites (3). The permeability of the PM is such that it either excludes or severely restricts access of ingested host antibodies to the

underlying digestive epithelial cells of the gut (3, 4). The PM, however, is bathed in ingested host antibodies and, therefore, PM proteins may be candidate vaccine antigens. Preliminary studies have shown that sheep vaccinated with crude PM protein extracts from the larvae of the fly *Lucilia cuprina* induce an anti-larval immune response (2, 5). These larvae feed on ovine tissue fluids, dermal tissue, and blood, eventually causing a severe cutaneous myiasis associated with considerable production losses in the sheep industry. In this study, we report the purification of a protein from *L. cuprina* larval PM that, when injected into sheep, induces an immune response that inhibits the growth of *L. cuprina* larvae that subsequently feed on serum from these vaccinated animals. The deduced amino acid sequence of this protein and the probable mechanism of action of the anti-larval immune response are also identified.

MATERIALS AND METHODS

Identification and Purification of Peritrophin 95. The isolation of PM by culture of *L. cuprina* larvae has been described elsewhere (5). A previous series of experiments demonstrated that a 4 M urea extract of detergent-washed PM solubilized a group of proteins (peritrophins) that, when injected into sheep, induced an anti-larval immune response measured by *in vitro* feeding and *in vivo* bioassays (5). The strategy for the isolation of one antigen, peritrophin 95, entailed successive protein fractionations that were each assessed for anti-larval activity in sheep vaccination trials. Peritrophin 95 was isolated and purified from a 6 M urea extract of detergent-washed PM by a two-step chromatographic procedure involving gel permeation chromatography on Superose 12 (Pharmacia) followed by Mono Q anion exchange chromatography (Pharmacia) (6). Both procedures were performed in the presence of 6 M urea. The yield of purified peritrophin 95 was 250 $\mu\text{g/g}$ (dry weight) of PM. Protein concentrations were determined using the Pierce BCA kit with BSA as a standard. The urea contained within buffers was sufficiently diluted to avoid interference with protein determinations. SDS/PAGE and biotinylated lectin blot analysis were carried out as described elsewhere (6).

Vaccination of Sheep with PM Proteins. Sheep (eight sheep per vaccination group), which had not previously suffered a cutaneous myiasis were initially injected with peritrophin 95 in the muscle of the rear leg and 4 weeks later in the muscle of the neck. The adjuvant for the first injection was Freund's complete adjuvant; the second injection used Freund's incomplete adjuvant (Sigma). Each sheep received a total of 63 μg of peritrophin 95. Two weeks after the second injection, the

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Abbreviations: PM, peritrophic membrane; NSS, normal sheep serum. Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. U23828).

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effect of vaccination was assessed by an *in vitro* larval growth bioassay that consisted of allowing first-instar larvae to feed on an agar-based medium containing serum from the vaccinated animals (7). The number of surviving larvae and their weights were measured after 20 h. There was no significant difference between the mean weights (and mean survival) of larvae feeding on control serum or individual prevaccination serum.

Isolation of Ig from Serum and Feeding of Concentrated Ig to *L. cuprina* Larvae. Total Ig was isolated from the serum of one of the strongest responding sheep and also from pooled sera from control sheep that had been injected with adjuvant and PBS (8). An antigen-capture ELISA was performed to determine the relative concentration of total Ig in the original control serum and after isolation (7). The Ig samples were concentrated and added to 4 ml of normal sheep serum (NSS) to give immune Ig concentrations equal to one, two, and four times those in the original immune serum. The total volume was adjusted to 5 ml with PBS and formulated into agar-based diets on which *L. cuprina* larvae were grown (7).

***In Vitro* Feeding of *L. cuprina* Larvae with Colloidal Gold.** Neonate larvae were allowed to feed for 5 h at 34°C on a diet containing NSS with a 4-fold enrichment of total Ig isolated from control serum or for 20 h on a similar diet with a 4-fold enrichment of immune Ig. The shorter feeding duration (5 h) adopted for control larvae ensured that these larvae were at a similar stage of development (first instar) to the larvae fed on immune Ig for 20 h. After being counted and weighed to confirm the enhanced larval growth inhibition caused by the Ig-enriched serum, some of the larvae were then transferred to the same diets but also containing 15% (vol/vol) of an aqueous suspension of colloidal gold (mean diameter, 6 nm) stabilized by conjugation to the B-chain of insulin (9). These larvae were allowed to continue feeding on these preparations for 2 h at 34°C. The larvae were then removed, carefully dissected open, and processed for transmission electron microscopy (9).

Peptide Amino Acid Sequences and Oligonucleotide Synthesis. The amino-terminal amino acid sequence of peritrophin 95 and internal peptide sequences were determined by published procedures (6). Peptide amino acid sequences were used to design degenerate oligonucleotide primers (Pharmacia LKB Gene Assembler Plus) suitable for PCR (10). The sense primer derived from the amino-terminal sequence and peptide 1 was 5'-C(TCA)AC(TCA)GG(TCA)AC(TCA)AA(AG)TT-(TC)CC(TCA)AG-3' and the antisense primer derived from peptide 3 was 5'-C(AG)AA(AG)TA(AGT)AT(AGT)GA(A-G)AA(AGT)AC(AGT)CC-3'. Bracketed nucleotides show alternatives at a specific position.

Cloning of cDNA Coding for Peritrophin 95. A peritrophin 95-specific double-stranded DNA probe was prepared by using PCR in conjunction with the oligonucleotide primers described above and *L. cuprina* first-instar larval cDNA. The reaction mixture contained 5 ng of cDNA, 500 ng of each oligonucleotide primer, all four dNTPs (each at 2 mM), 4 mM MgCl₂, 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Cetus) in 100 μl of 10 mM Tris·HCl (pH 8.3) and 50 mM KCl. Amplification was performed with the following conditions: 1 cycle of 5 min at 95°C, 1 min at 50°C, and 5 min at 72°C; 33 cycles of 1 min at 95°C, 1 min at 50°C, and 5 min at 72°C; and 1 cycle of 1 min at 95°C, 1 min at 50°C, and 10 min at 72°C. A single DNA product (595 bp) was amplified and sequenced on both strands (11). The DNA product was labeled and used to screen a *L. cuprina* first-instar cDNA library constructed in λgt-11 (6). Seven positive plaques were purified. A 1,616-bp DNA insert from one clone was excised and sequenced on both strands (11, 12). DNA inserts from three additional positive clones were sequenced and contained fragments of the sequence derived from the first clone.

RESULTS

Purification of Peritrophin 95. Preliminary experiments using *L. cuprina* larval PM demonstrated that it induced an immune response in vaccinated sheep that inhibited the growth of larvae that subsequently fed on serum from those sheep. A series of protein fractionations using vaccination trials in sheep as a bioassay were undertaken to purify individual PM proteins responsible for inducing the anti-larval effects. With this bioassay, a 95-kDa integral PM protein (peritrophin 95) was purified from a 6 M urea extract of detergent-washed PM by using a combination of gel-permeation and anion-exchange chromatography (Fig. 1*a*). The apparent size of peritrophin 95 measured by SDS/PAGE varied according to the percentage of polyacrylamide ($M_r = 80,000 \pm 8,000$ after extrapolation to 0% polyacrylamide). Fig. 1*b* shows that peritrophin 95 binds biotinylated wheat germ lectin. This interaction was inhibited by incubation of the lectin blot with 0.3 M GlcNAc. Thus, peritrophin 95 contained oligosaccharides with terminal GlcNAc (or NeuAc) sugars. Weak binding was observed with biotinylated lentil lectin; however, *Phaseolus vulgaris* E lectin, peanut agglutinin, *Sophora japonica* lectin, and *Pisum sativum* lectin did not bind (results not shown).

Anti-Larval Effects of Serum from Sheep Vaccinated with Peritrophin 95. Peritrophin 95 induced an anti-larval immune response in sheep as assessed by the ability of serum from vaccinated sheep to inhibit growth of *L. cuprina* larvae in an *in vitro* feeding bioassay (Fig. 2). There was a mean larval weight reduction of $60 \pm 16\%$ ($P < 0.001$) compared with the mean weight of larvae feeding on control serum. There was no significant effect on the survival of larvae. The effect on larval weights is a specific immune response to peritrophin 95 because a number of purified proteins from several diverse species, including *L. cuprina*, as well as several crude protein extracts from *L. cuprina* larvae, *Boophilus microplus* adults and larvae, *Escherichia coli*, and cultured insect cells did not induce anti-larval effects in serum from appropriately vaccinated sheep (results not shown).

Enhanced Anti-Larval Effects with Serum Enriched with Anti-Peritrophin 95 Ig. Total Ig was isolated from the serum of a strongly responding sheep vaccinated with peritrophin 95 and from pooled sera from control sheep that received adju-

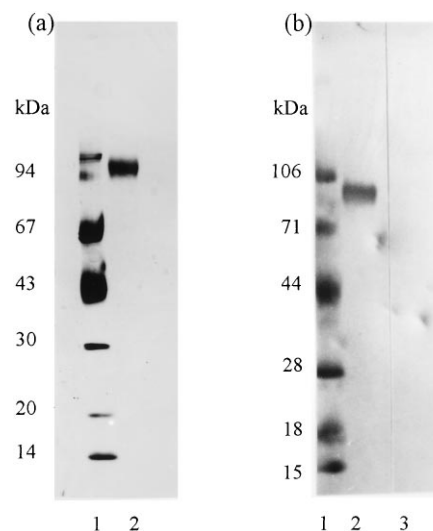


FIG. 1. SDS/PAGE of purified peritrophin 95. (a) Silver-stained SDS/PAGE of peritrophin 95 (reduced). Lanes: 1, standards; 2, peritrophin 95 (2 μg). (b) Biotinylated lectin blot of purified peritrophin 95 (5 μg, lanes 2 and 3). Lanes: 1, standards; 2, biotinylated wheat germ lectin; 3, biotinylated wheat germ lectin after incubation of the filter with 0.3 M GlcNAc.

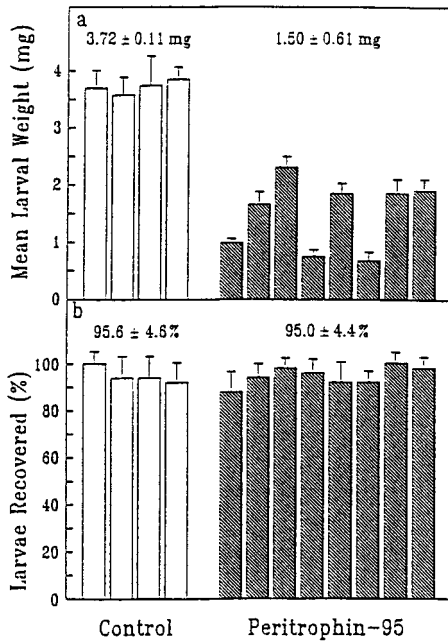


FIG. 2. Anti-larval effects of serum from sheep vaccinated with peritrophin 95. The effects of anti-peritrophin 95 serum from eight sheep on the mean weight (*a*) and mean survival (*b*) of *L. cuprina* larvae were measured (shaded histograms) by using an *in vitro* feeding bioassay. The unshaded histograms refer to corresponding results for a control group of four sheep. Vertical bars on histograms indicate 1 SD. Numbers above the control and peritrophin 95 groups represent group means \pm 1 SD. The mean weight of larvae feeding on the serum from sheep vaccinated with peritrophin 95 was significantly different from the control group ($P < 0.001$).

vant alone. The isolated Ig was reconstituted into NSS at 1-fold, 2-fold, and 4-fold enrichments as measured by ELISA. Results of the *in vitro* larval feeding bioassay undertaken with these modified sera are shown in Fig. 3. The original anti-larval activity in the immune serum resulted in $85 \pm 2\%$ mean larval weight reduction. Transfer of total Ig from this serum into NSS at the original Ig concentration (1 \times Ig) caused $70 \pm 7\%$ mean larval weight reduction compared with the corresponding control. Thus, the anti-larval activity was mediated by Ig. There was enhanced inhibition of larval growth when the level of anti-peritrophin 95 Ig in NSS was increased. The mean larval weight reduction was $70 \pm 7\%$ at 1 \times Ig, $86 \pm 9\%$ at 2 \times Ig, and $98 \pm 3\%$ at 4 \times Ig compared with corresponding controls. At the latter Ig concentration, a mean of $52 \pm 25\%$ of the larvae were not recovered compared with the corresponding value of $2 \pm 4\%$ for the appropriate control. Reduced larval recovery was observed only when the mean larval weight reduction of the surviving larvae was at least 80% (ref. 13 and unpublished results). Heating of the immune serum for 30 min at 55°C did not alter the anti-larval activity, indicating that complement was not involved (results not shown). The high Ig concentration introduced into NSS caused some inhibition of larval growth in control experiments (but had no effect on larval survival). However, this effect was relatively small compared with effects observed with serum enriched with immune Ig. For example, the control mean larval weight at 4 \times Ig was 48% of the value for the control at 1 \times Ig; however, the corresponding level of anti-peritrophin 95 Ig reduced the mean larval weight by 95%.

Effect of Immune Serum on Larval PM. Visual and microscopic examination of the small larvae feeding on immune serum did not reveal any external signs of damage. One possibility was that the ingested antibodies were binding to peritrophin 95 on the PM and interfering with the movement of nutrients through this semipermeable membrane, thereby

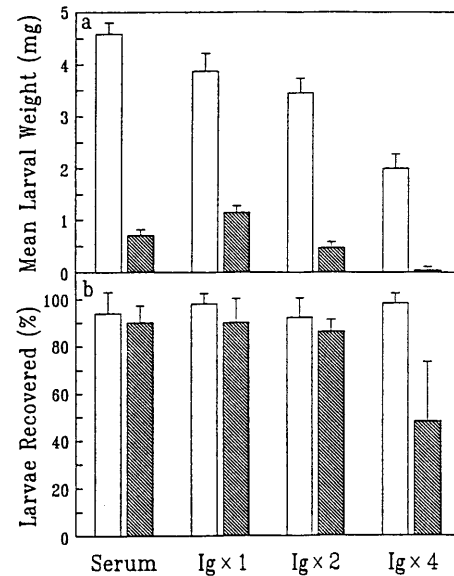


FIG. 3. Enhanced larval growth inhibition using serum enriched with Ig isolated from a sheep vaccinated with peritrophin 95. Larvae were allowed to feed on the Ig-enriched serum for 20 h in an *in vitro* feeding bioassay after which mean weight (*a*) and mean survival (*b*) were measured. Controls, Ig from control sheep supplemented into NSS at 1-, 2-, and 4-fold enrichments (unshaded); Ig, (anti-peritrophin 95) immune Ig supplemented into NSS at 1- (1 \times Ig), 2- (2 \times Ig), and 4- (4 \times Ig) fold enrichments (shaded); serum, original unmodified anti-peritrophin 95 serum. Error bars denote 1 SD.

limiting the availability of nutrients to the digestive gut epithelia. To test this possibility, neonate larvae were fed on NSS enriched 4-fold with immune Ig. This enriched serum reduced larval weights by greater than 90% in an *in vitro* feeding bioassay. Also included in the Ig-enriched diet for the last 2 h of growth were 6-nm colloidal gold particles. The electron micrograph shown in Fig. 4*a* demonstrates that the colloidal gold particles ingested by a larva feeding on NSS enriched 4-fold with control Ig were present within the PM and freely distributed among the microvilli of the digestive epithelial cells lining the gut. A similar pattern was seen in larvae allowed to grow on this preparation until second instar. These results indicate that particles of this size can freely traverse the PM and are consistent with other studies that indicate that the permeability limit for the PM is ~ 10 nm (unpublished data). In contrast, in a larva fed on immune Ig-enriched serum, the gold particles were almost completely restricted to the endoperitrophic space (Fig. 4*b*). Very few gold particles ($<0.5\%$) were found within the PM or associated with the digestive epithelial cells. Further, there was now a new layer lining the gut lumen side of the PM that almost totally excluded the ingested gold particles from access to the PM. This layer, of undefined composition, extended from the anterior midgut through the midgut to the hindgut and was in places 1 μ m thick compared with a PM thickness of 70–100 nm.

cDNA and Deduced Amino Acid Sequences of Peritrophin 95. Fig. 5 shows the nucleotide and deduced amino acid sequences of cDNA coding for peritrophin 95. The cDNA sequence (1,616 bp) contained an ORF of 1,443 bp that coded for a polypeptide of 480 amino acids containing all peptides derived from peritrophin 95 (Fig. 5). The deduced amino acid sequence of peritrophin 95 contains an amino-terminal signal sequence of 24 amino acids and a mature polypeptide of 456 amino acids. The mature protein has a pI^{calc} of 4.5 (4.7 ± 0.2 by isoelectric focusing) and a calculated M_r of 50,117, which is significantly different from that measured by SDS/PAGE ($M_r = 80,000 \pm 8,000$). The sequence contains 30 Cys residues that are clustered in the amino-terminal two-thirds of the

using semipermeable membranes that become blocked by partial solute precipitation or solute polarization (29). Analogous effects on larval growth and the same type of induced layer on the PM occur in larvae fed on wheat germ lectin or lentil lectin, which also bind to PM proteins (9). The data indicate a similar mechanism of action and reinforces the proposal that the anti-larval effects are mediated by a biophysical effect involving the formation of an impervious layer on the gut lumen side of the PM triggered by the binding of proteins to the PM that block pores in the PM.

The structure of peritrophin 95 suggests that it has the capacity to bind chitin within the PM and has a mucin-like structure in the carboxyl-terminal one-third of the protein that may be extensively glycosylated and probably highly exposed in the gut lumen. The ability of peritrophin 44 (6) and potentially also peritrophin 95 to bind chitin, a linear polymer of GlcNAc, raises another interesting possibility that these proteins could form a coordinated network in the PM mediated by interactions between the chitin-binding domains of these proteins and oligosaccharides containing GlcNAc present on peritrophin 95. These interactions could occur in addition to the direct binding of these proteins to chitin fibrils present in the PM. It is possible that these combined interactions dictate pore size. The reduction in PM permeability after the binding of antibodies to peritrophin 95 is consistent with this model.

The extension of this potential immunocontrol strategy to the control of other insects that cause cutaneous myiasis or insects feeding directly on mammalian blood will be dictated by the specific physiological characteristics of these insects, particularly the intimacy of their relationship with their mammalian hosts. The key aspect of this potential control strategy is the delivery of large quantities of antibody directed against the PM of the insect. The ability to produce relatively large quantities of functional recombinant antibodies in transgenic plants (30) raises the intriguing possibility that this potential immunocontrol strategy could also be used to control insect pests of plants.

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