Biological control of flystrike: use of live bacterial vectors to deliver insecticidal proteins and insecticidal double stranded RNA.

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Summary

We propose a new method for the control of flystrike. This method would utilise live bacteria to deliver insecticidal proteins and/or dsRNA to fly larvae, thus killing the larvae. Live vectoring has been widely used to deliver vaccine antigens and there are some reports of this type of delivery system being used to deliver other biologicals. The adaptation of this technology to flystrike will require the choice of suitable vector bacteria. A review of the literature indicates a number of possible choices. A variety of insecticidal biological molecules are discussed.

Keywords

Live vector, insecticidal proteins, RNA interference, recombinant antibodies, biological control

Introduction

A primary focus in flystrike management is reducing the use of contaminating chemical insecticides. The problems associated with the use of synthetic chemical insecticides currently applied for control are declining efficacy arising from resistance and the creation of residue problems in the wool and at dip sites, wool scours and carpet dye baths. The broad spectrum activities of organophosphates and synthetic pyrethroids pose real problems for the environment and public health. Such problems threaten the clean and natural image of wool products, and consequently also their markets. In order to reduce the use of chemical insecticides and hence reduce chemical residues, it is necessary to both improve management practices and to have alternative, non-chemical methods to tackle flystrike. Biological based methods of control are desirable.

Live vectors have been used to deliver a range of biological molecules. Considerable research has been directed towards the delivery of heterologous vaccine antigens and cytokines by live viruses and bacteria. Live bacterial vectors have also been used to deliver DNA vaccines. We propose that live bacteria could also be used as vectors to deliver a range of insecticidal biological molecules with the potential to assist in the control of flystrike. In particular they could deliver insecticidal proteins and peptides, insecticidal double stranded RNA, and anti-larval recombinant antibodies. Our concept is to develop an effective, easy to apply, environmentally friendly, biological method of controlling flystrike.

Bacterial Live Delivery Systems

A variety of different bacteria have been used as live delivery vectors. Attenuated pathogens such as *Salmonella typhimurium* (Curtiss *et al*., 1988), *Vibrio cholerae* (Chen *et al*., 1998), *Brucella abortus* (Comerci *et al*., 1998), *Mycobacterium bovis* BCG (Langermann *et al*., 1994), *Listeria monocytogenes* (Frankel *et al*., 1995), *Shigella flexneri* (Noriega *et al*., 1996), *Aeromonas salmonicida* (Noonan *et al*., 1995), and *Corynebacterium psuedotuberculosis* (Moore *et al*., 2000) have been used both as live vaccines against the diseases caused by the parent strains and as vectors to deliver heterologous, recombinant vaccine antigens in both humans and animals. Normal commensal bacteria such as *Streptococcus gordinii* (Medaglini *et al*., 1995) and non-pathogenic bacteria such as *Lactobacillus casei* (Maassen *et al*., 1999), and *Lactococcus lactis* (Norton *et al*., 1997) have also been investigated as vectors for the delivery of vaccine antigens.

A wide variety of antigens have been delivered using live bacterial vectors including many bacterial antigens, viral antigens (Karem *et al*., 1997; Choi *et al*., 2000), allergens (Vrtala *et al*., 1995), antifertility targets (Zhang *et al*., 1997), amoeba antigens (Ryan *et al*., 1997), tumour antigens (Pan *et al*., 1995) and parasite antigens (Gentschev *et al*., 1998; Matsumoto *et al*., 1996). In addition, a number of the attenuated pathogens have been used to deliver plasmid DNA for genetic vaccination (Jain and Mekalanos, 2000;

Paglia *et al*., 1998) and also cytokines to modify immune responses (Whittle *et al*., 1997; Saltzman *et al*., 1997).

It is clear that a wide range of potential live vector bacteria have been developed and tested. Whilst the major focus has been the delivery of antigens to induce an immune response there has also been some work on the delivery of biologically active molecules, e.g. the delivery of cytokines. There is a large body of work reported in the literature that indicates that live vectoring can be an effective way to deliver proteins.

Insecticidal Proteins

There are many reports in the literature of proteins with insecticidal activity. By far the most widely studied are the endotoxin proteins from *Bacillus thuringiensis* (Schnepf *et al*., 1998). A range of wild-type *B. thuringiensis* strains are used in commercial products for pest control (Navon, 2000) and the genes encoding various forms of the toxins have been introduced into a variety of plants to produce transgenic plants with resistance to major insect pests (Jouanin *et al*., 1998). A number of *B. thuringiensis* strains have been identified as being toxic to *Lucilia cuprina* (Lyness *et al*., 1994) and although some of these have been tested as control measures on sheep they are not being progressed further because of various problems. There is still certainly scope to further investigate the use of Bt toxins in the control of flystrike – what is needed are more effective and more specific toxins and better delivery mechanisms.

There are many other proteins that have been reported to have insecticidal activity (Table 1). These include lectins, chitinases, cholesterol oxidases, protease inhibitors, cuticle degrading protease, enterlobin, α-amylase inhibitors, venom derived peptide toxins, chaperonin, ribosome inactivating proteins, and lipoxygenases (Table 1).

Table 1. Insecticidal proteins.

Protein	Insects affected	References
Lectins, e.g. Snowdrop lectin	Aphids, leafhoppers, moths	Down *et al*., 1996; Fitches *et al*., 1997; Powell *et al*., 1993
Chitinases	Anopheles, Spodoptera, Heliothis	Kramer & Muthukrishnan, 1997
Cholesterol oxidases	Cotton boll weevil, budworm	Corbin *et al*., 1994
Protease inhibitors	Various lepdoptera, coleoptera	Hilder and Boulter, 1999
Cuticle degrading protease	Tobacco hornworm	St.-Leger *et al*., 1992
Enterolobin	Coleoptera	Sousa *et al*., 1993
α-amylase inhibitors	Coleoptera	Ishimoto and Kitamura, 1989
Venom derived peptide toxins, e.g.		
Tx4(6-1)	Flies, cockroaches	Figueiredo *et al*., 1995
BmK IT_4	Cotton bollworm	Jiang *et al*., 2001
ω-atracotoxin	Blowflies, bollworms, etc.	Wang *et al*., 1999
Chaperonin	Cockcroach	Yoshida *et al*., 2001
Hirsutellin A (Ribosome inactivating protein)	Waxmoth, mosquito	Mazet and Vey, 1995
Lipoxygenase	Tobacco hornworm	Shukle and Murdock, 1983

The potency and specificity of most of these insecticidal proteins is difficult to judge from the information in the published literature. Most of the work has been directed towards the control of crop pests, so the insect species reported to be susceptible reflect this bias. It is likely that a number of the above proteins, or closely related proteins, will have insecticidal activity against *L. cuprina*.

It is also possible that the vectored delivery of insect peptide hormones could be used to cause perturbations in *L. cuprina* growth and development. Hormones such as diuretic hormone, juvenile hormone and ecdysteroid modifying enzymes, diapause hormone, culekinin depolarizing hormone, allatostatins, prothoracicotropic hormones and eclosion hormones could all be potentially used. Hormones may have the advantage that lower concentrations of the active protein may be effective.

Insecticidal RNA

In the last few years it has been demonstrated in a number of organisms that double-stranded RNA can be used to specifically silence gene expression. This finding, termed RNA interference (RNAi), has been most widely used to study gene function in the nematode *Caenorhabditis elegans* (Fraser *et al*., 2000), but has also been used in planaria (Sanchez Alvarado and Newmark, 1999), hydra (Lohmann *et al*., 1999), trypanosomes (Ngo *et al*., 1998), zebrafish (Li *et al*., 2000), mice (Bahramian and Zarbl, 1999), and *Drosophila* (Kennerdell and Carthew, 1998; Misquitta and Paterson, 1999).

Of particular relevance is the finding that dsRNA can cross cell boundaries. It has been demonstrated in *C. elegans* that specific gene interference can occur when the nematodes ingest bacteria expressing dsRNA complimentary to a nematode gene (Timmons and Fire, 1998). Hence, as RNAi has been shown to be effective in *Drosophila*, we would postulate that it might be possible to affect *Lucilia cuprina* viability by delivering dsRNA with live bacteria seeded onto sheep fleece. The dsRNA would be directed towards silencing an essential gene function - there are many genes that could potentially be targeted.

Recombinant Antibodies

There are many examples in the literature of the bacterial production of active recombinant antibody fragments (Little *et al*., 1995). Such proteins have the potential to be delivered by live bacterial vector systems, just like any other protein. The technology for the *in vitro* identification and cloning of single chain antibodies (scFv) with a desired binding specificity is well established (Hoogenboom and Chames, 2000). We postulate that it may be possible to produce vector bacteria expressing scFv that could reduce the viability of *L. cuprina* larvae by either sequestering essential digestive enzymes or by physically blocking the gut lumen.

Vector Bacteria

The choice of bacteria to develop as the live delivery vehicle for any of the protein or RNA molecules would be directed by three main considerations. (1) It should be maintained in the fleece of sheep for a considerable length of time, (2) ideally it should also be able to establish, maintain, and proliferate itself in the gut of *L. cuprina* larvae, and (3) it should be able to make sufficient amounts of the active biological molecules to retard or stop larval development.

There is only a very limited amount of information available about the microbial ecology of sheep fleece, most collected as part of investigations of susceptibility of sheep to fleece-rot (Chin and Watts, 1992). The major types of bacteria present appear to be various *Pseudomonas* and *Bacillus* species (Lyness *et al*., 1994) but *Stapylococcus* and *Micrococcus* species are also frequently found along with a range of less commonly identified bacteria. At least some members of these groups of bacteria can be easily manipulated and genetically engineered to produce recombinant proteins so in this regard they are attractive candidates as live vectors. Our laboratory has worked on *Corynebacterium pseudotuberculosis*, a pathogen of sheep that is common in the environment. We have constructed non-pathogenic, rationally attenuated versions of this bacterium which have been developed as live vectors for the delivery of vaccine antigens (Hodgson *et al*., 1992; Moore *et al*., 2001). It is possible that these strains could also be used to deliver insecticidal proteins. Earlier attempts to use *Bacillus thuringiensis* as a live control mechanism for flystrike were frustrated, in part, because the bacteria were not maintained on the sheep in sufficient numbers for a sufficient length of time. A particularly interesting prospect is to use some of the Pseudomonas species which rapidly multiply in the wet, humid conditions in which flystrike is most prevalent. The idea would be that they could be maintained in the fleece in relatively low numbers in drier conditions but rapidly multiply to meet the fly challenge when the fleece humidity increases - a natural, appropriate dosing scheme!

The microbial ecology of the gut of *L. cuprina* larvae is unknown. Therefore, without some basic survey work being performed, it is difficult to postulate what sort of bacteria might be appropriate for colonising the gut. It is also unknown if any of the bacteria that can populate the fleece could also colonise the larval gut. Central to our basic concept is the view that blowfly larvae grazing on the skin surface of sheep will

inevitably pick up bacteria from the skin. We would assume that some of these bacteria could survive in the larval gut, even if only transiently.

Product Profile

Our vision is to develop a biological product for the control of flystrike that is easy to apply and long lasting. It would consist of a live bacterial vector engineered to produce proteins, peptides or dsRNA deleterious to *L. cuprina*. The vector would be derived from a bacterial species that is normally resident in sheep fleece and would be chosen on the basis of its persistence in the wool and on the skin of sheep. Ideally the vector would also be capable of persisting in the gut of *L. cuprina* larvae. The bacteria expressing insecticidal biological molecules would be ingested by fly larvae, which would subsequently die or have retarded development.

Other Applications

This approach, of live vectoring of biologically active molecules, has the potential to be used for a variety of other animal health applications. For example, we are currently investigating the delivery of antimicrobial proteins for the treatment of gut diseases. The approach indicated for flystrike could also be applied to the control of gut nematodes and for this application the choice of vector bacteria may be easier.

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