ANTI-INSECT ANTIBODIES: A NOVEL APPROACH FOR CONTROL OF PHYTOPHAGOUS INSECT PESTS

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CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

Introduction

In 1962, Rachel Carson's Silent Spring heightened concerns about the impacts of chemical pesticides. Since that time public opinion and government regulation have changed the trend in agriculture from one in which pesticides were used as the sole means of insect control to the more integrated control programs of today (Pedigo, 1989). Although the trend is toward less frequent use of pesticides, chemical control of arthropods is still a source of public concern. Concerns range from pesticide impact on the environment and water quality, to toxic effects on non-target species, including humans, to the future impact of pesticide-resistant pests and pathogens.

Elimination of the use of chemical pesticides is not realistic at this time, but an integrated approach to the management of pest species is advocated, if not demanded, to reduce our reliance on chemical control. Insect pest management strategies stress the use of multiple tactics including; the judicious use of pesticides, crop rotation, field sanitation, utilization of predator and parasitoid populations, and the use of insect-resistant crop plants to reduce and maintain pest populations below levels of economic damage while incurring minimal environmental impact (Pedigo, 1989). Within the framework of pest management strategy, an effective and environmentally friendly method is the use of insect-resistant crop plants (Pedigo, 1989).

The use of insect-resistant crop plants offers many advantages over chemical control alone (Gatehouse et al., 1992). The factors imparting resistance are contained on or within the plant throughout the entire growing season. Target insects are affected at the most susceptible stage of development, regardless of weather conditions. Internal insect resistance factors protect against root feeding, or boring, insects that are difficult to reach with standard chemical treatments, and the risk of leeching toxic chemicals into groundwater supplies is eliminated.

There are, however, disadvantages to the development and use of insect resistant plants. Many of the traditional sources of resistance may have decreased potency, or become wholly ineffective in the future, due to the selection of resistance breaking populations (Pedigo, 1989; Cantelo, 1992). Selective pressure on a pest population by a resistant cultivar with a single gene for resistance, monogenic resistance, may induce an adapted pest population called a biotype. Selection for resistant biotypes may be reduced by developing cultivars with multiple resistance genes to a single insect species, a method known as polygenic or horizontal resistance (Smith, 1989). Polygenic resistant cultivars are difficult to produce using traditional breeding methods. Development of a single-gene or monogenic cultivar that is resistant to a single pest species may take three to five years, and polygenic resistance to one pest species may take several years longer (Smith, 1989).

Recombinant DNA and genetic transformation technologies have provided new opportunities for broadening the repertoire of properties imparting insect resistance to crop plants. Genetic transformation has two major advantages over traditional plant breeding practices alone (Gatehouse et al., 1992). The first is the ability to transfer a

single gene or multiple genes for resistance to recipient plants without transferring undesirable traits. This greatly reduces the time needed to produce a resistant cultivar. Secondly, genes from alternate sources can be used. In traditional plant breeding, only genes from closely related plants can be combined, while with plant transformation, resistance genes from unrelated plant species or even animals can be utilized (Gatehouse et al., 1992).

A growing number of insect-resistant plants have been genetically modified to produce insecticidal proteins, and the number of insecticidal genes available is steadily increasing (Gatehouse et al., 1992). Several categories of insecticidal proteins have been discovered, and they have been expressed in genetically manipulated plants to confer insect resistance. These include the insecticidal crystal proteins of *Bacillus thuringiensis* Berliner, plant lectins, proteinase inhibitors, enzymes and antibodies (Estruch et al., 1997; Gatehouse et al., 1992; Boulter, 1993; Lal and Lal, 1993).

Although antibodies have yet to be shown to have insecticidal properties toward phytophagous insect species, antibody-based control has been identified for several species of arthropods parasitic on mammals (Wikel, 1988; Wikel et al., 1992; Willadsen et al., 1993; Opdebeeck, 1994). Animals immunized with an antigen preparation of the parasite, or its tissues, produce antibodies specific for the antigens present in the immunization preparation. When the parasitic species feeds on the immunized animal, the antibodies in the meal bind to their specific antigens in the parasite. Antibody binding neutralizes the antigen or prepares the attached cell for destruction, severely impairing the biology of the parasite. This generalized procedure has been effective

against lice, fleas, mosquitoes, myiasis causing flies, and several species of ticks (Wikel, 1988; Wikel et al., 1992; Willadsen et al., 1993; Opdebeeck, 1994; Wikel et al. 1996).

Insecticidal potential of antibodies is well established, and techniques exist for genetically transforming plants to produce insecticidal antibodies. Plants do not naturally produce antibodies, but contain the mechanisms required for protein manufacture and assembly. Fully functional mammalian antibodies and functional antibody fragments have been expressed in plants (Smith, 1996). Production of these proteins may be targeted to any plant organ or subcellular compartment (Hiatt 1990), as well as, intercellular spaces (DeWilde et al., 1996). Ability to express antibodies in plants may have great potential for pathogen and pest resistance in crop plants, and this approach has already been used successfully against infection by a plant virus (Tavladoraki et al., 1993). Most crop plant species, however, have never been transformed to express antibodies; and to date, no published studies have been performed to determine the possibility of developing antibody-producing insect-resistant plants.

The purpose of this research was to determine if protective antibodies could be formed against a phytophagous insect species. Genes for anti-insect antibodies could then be engineered for future expression in transgenic plants. This would provide a novel approach to plant protection.

The pea aphid, *Acyrthosiphon pisum* (Harris) (Homoptera: Aphididae) was chosen for this research for several reasons. First, pea aphids are easily maintained on artificial diet preparations (Febvay et al., 1992; Abisgold et al., 1994; Akey and Beck, 1972). Secondly, pea aphids normally feed in the phloem of the plant where few proteins are ingested and thus lack gut endoprotease activity (Srivistava and Auclair, 1963; Rhabe

et al., 1995). Due to the lack of gut endoproteases, pea aphids would be unlikely to digest an ingested antibody. Finally, pea aphids are relatively large aphids which allows for easier handling and dissection and a more rapid accumulation of midgut material.

Research Hypothesis and Specific Aims

Hypothesis

Polyclonal mammalian antibodies produced against proteins present in the midgut of pea aphids will cause detrimental effects upon ingestion by aphids.

Specific Aims

The following specific objectives were addressed:

1. Create a bioassay system using artificial diets to evaluate effects of ingested antibodies on the pea aphid.
2. Generate and characterize rabbit antibodies against proteins comprising structure and/or contents of the midgut of the pea aphid.
3. Determine the effects of antibodies against midgut proteins on survival, development, and reproduction of the pea aphid.

Review of Literature

The Pea Aphid

The pea aphid, is a pest of world-wide economic importance (Harper et al., 1978). This aphid has a wide range of leguminous hosts and ornamental species on which it feeds. It can be found on forages such as vetch, clovers, and alfalfa, as well as, peas, beans, and leguminous weeds.

Pea aphids quickly build up large colonies on plants via parthenogenetic reproduction. Large populations of pea aphids feeding in the phloem of plants cause chlorosis, wilting, stunting, and eventually plant death. Pea aphid infestations have been shown to reduce cold hardiness (Harper and Freyman, 1979) and yields and quality of alfalfa (Wilson and Quisenberry, 1986), plant growth rates (Barlow and Messmer, 1982) and seed weights of field peas (Soroka and Mackay, 1990). Pea aphids are also important vectors of many plant viruses (Harper et al., 1978).

Currently, methods for the control of pea aphids are predominantly chemical insecticides and traditional (non-transgenic) insect-resistant plants. Traditional plant resistance to pea aphids has been reported for several plant species, including field peas (Soroka and Mackay, 1991), alfalfa (Febvay et al., 1988; Dreyer et al., 1987), red clover (Gorz et al., 1979), and white clover (Markkula and Roukka, 1971). Traditional plant resistance mechanisms may be overcome in the future by resistance breaking biotypes of pea aphids (Auclair, 1978; Cartier, 1963).

Overview of Plant Resistance to Insects

Plant resistance to insects can be generally defined as the genetically heritable ability of certain plants to sustain a lower level of damage than susceptible plants when attacked by a pest population (Smith, 1989). All plants have varying degrees of resistance to the insects and diseases that attack them, but none are totally resistant. Many crop varieties used today lack sufficient resistance to pests and disease-causing agents (Smith, 1989).

Plant resistance to insects has been classified according to different mechanisms or components. Painter (1951) used the terms tolerance, non-preference (antixenosis), and antibiosis to describe these mechanisms of resistance. Tolerance is described as an enhanced ability to withstand damage caused by a pest population or recover faster from damage than susceptible plants. Plants possessing antixenosis may lack the appropriate attractant stimuli necessary for a particular species to feed, so that the insect seeks another host. The third category of insect resistance is antibiosis. Antibiosis occurs when the insect pest experiences a direct negative effect on its biology due to an association with the resistant plant. Instances where the plant kills the insects, delays development, reduces fecundity, or modifies behavior in such a way as to cause them to be more susceptible to other biotic or abiotic pressures, may be classified in this category. All three mechanisms of resistance may exist in a single plant cultivar.

Use of insect resistant crop plants has many benefits due to compatibility with chemical, biological, and cultural control methods (Smith, 1989). By definition, insect-resistant crop plants have a greater productivity than susceptible plants when initially placed under an equal pest burden. If conventional insecticides are utilized, fewer applications may be necessary to achieve appropriate levels of control, as based on

economic thresholds. Use of insect-resistant plants may also enhance effectiveness of entomophagous parasitoids and predators. Certain resistance factors may slow movement or response time of the prey, thus decreasing search time and increasing search efficiency of the predator or parasitoid. Use of insect resistant plants is also compatible with cultural control methods, such as crop rotation and sanitation. When both are used together their effects are additive. Finally, the use of insect resistant plants can also aid indirectly in prevention of disease spread by reducing abundance of competent insect vectors (Maramorosch, 1980).

Control of Invertebrate Pests Using Antibodies

Utilization of antibodies and their properties for control of invertebrates that are parasitic to mammals has great potential and thus far has been quite successful (Wikel, 1988; Wikel et al., 1992; Willadsen et al., 1993; Opdebeeck, 1994; Wikel, 1996). Immunization of animals with parasite midgut and partial- or whole-body antigen preparations have been shown to cause negative effects in several groups of parasitic invertebrates feeding in or on immunized animals. Immunization with parasite antigens has been shown to be effective against helminths, insects, and ticks.

Stomach worms, *Haemonchus contortus* (Rud.), recovered from sheep immunized with contortin, a digestive tract protein of the stomach worm, weighed less than worms recovered from unimmunized sheep (Munn et al., 1987). Immunization of merino lambs with *H. contortus* extracts enriched with protein H11, the major microvillar integral membrane protein of *H. contortus* intestine, was effective in reducing parasite egg production and numbers of third stage stomach worm larvae (Munn et al., 1993). The

immunizations resulted in an 88% reduction in the total worm count as well as an 89% reduction in egg production.

Immunization of animals with insect-associated antigens has potential for control of endo- and ectoparasitic insects. Protective immunity has been induced against sucking lice, mosquitoes, and myiasis causing flies (Opdebeeck, 1994). Human body lice, *Pediculus humanus humanus* Linnaeus, reared on rabbits that were immunized with whole body or midgut homogenates of these lice took smaller blood meals than those on control rabbits and had reduced survival, slower development, and lower fecundity (Ben-Yakir and Mumcuoglu, 1988). Many lice reared on midgut immunized rabbits died due to rupture of the gut wall. Ratzlaff and Wikel (1990) found that mice immunized with whole louse homogenate acquired resistance against subsequent lice infestations. Similar results have been found for the mosquito species: *Aedes aegypti* Linnaeus (Hatfield, 1988; Ramasamy et al., 1988), *Anopheles tessellatus* Theobald (Ramasamy et al., 1992), and *Anopheles stephensi* Liston (Alger and Cabrera, 1972). Reduced viability and/ or fecundity of feeding mosquitoes were caused by anti-mosquito immunization.

Larvae of the sheep blowfly, *Lucilia cuprina* (Wiedemann), were reared on serum from sheep which had been immunized with the PM44 antigen from the peritrophic membrane of sheep blowfly larvae (Willadsen et al., 1993). Blowfly larvae which fed on the serum experienced a 60 to 90 percent decrease in growth versus control larvae. Resistance to cattle grub larvae, *Hypoderma lineatum* (Villers), has also been induced in cattle using Hypodermin A antigen, purified from first instars (Pruett et al., 1987). Many other insects that are parasitic on mammals may be good candidates for using an immunization type approach for control (Wikel et al., 1992).

Use of antibody-mediated protective immunity has been very successful against ticks (Wikel, 1996). Trager (1939) first reported induced immunity to ticks. Guinea pigs immunized with an extract of whole *Dermacentor variabilis* (Say) larvae were resistant to subsequent infestation by larvae. Immunization of animals with extracts of internal organs of ticks including salivary gland, reproductive organs, and midgut also provide protection against ticks (Wikel et al., 1992). Allen and Humphreys (1979) immunized guinea pigs with homogenates of *Dermacentor andersoni* Stiles midgut and reproductive tract or a homogenate of all internal organs. Adult ticks that fed on guinea pigs immunized with the homogenate of all internal organs did not complete engorgement or produce eggs. Ticks fed on the animals immunized with the reproductive tract and midgut homogenate did produce eggs, but none were viable. Wikel (1988) immunized guinea pigs with antigens of *Amblyomma americanum* (Linnaeus) digestive tract. When ticks were fed on the immunized guinea pigs, engorgement weights were reduced by 54 to 60 %, and frequent mortality during engorgement was attributed to damage to the gut. Rats immunized with midgut of *D. variabilis* also showed immunological resistance to tick infestations (Ackerman et al., 1980).

The most successful use of protective antibodies against ticks has been in the pursuit of an anti-tick vaccine against the cattle fever tick, *Boophilus microplus* (Canestrini). Johnston et al. (1986) immunized cattle with an extract of engorged *B. microplus* females which caused a 70 percent reduction in tick fecundity and a 90 percent reduction in tick infestation. Damage to the gut of the ticks was reported to be the primary source of protection in this study (Agbede and Kemp, 1986). Opdebeeck et al. (1988) used membrane-associated antigens of *B. microplus* midgut to induce protection

against ticks. Upon further study, three membrane bound antigens were isolated that accounted for much of the effect of the antigen preparation (Wong and Opdebeeck, 1989).

From a crude suspension of tick organs of semi-engorged *B. microplus* females, Willadsen et al. (1988) isolated a protective antigen fraction by detergent extraction, gel permeation chromatography, and preparative isoelectric focusing. This fraction was found to reduce infestation of *B. microplus* larvae. Willadsen et al. (1989) purified a membrane bound glycoprotein, Bm86, which acted as a highly protective antigen. Immunization with microgram quantities of Bm86 were sufficient to protect cattle from infestation by larvae. Recombinant Bm86 antigen was then produced in *Eschericia coli* (Migula) and baculovirus systems, and the manufactured antigen maintained its protective antigenicity (Tellam et al., 1992). Recombinant Bm86 produced in yeast was used to confer protection against several other strains of *B. microplus* (Penichet et al., 1994). In a more recent study, the monoclonal antibodies, BrBm 1, against different instars and tissues, and BrBm 2, against antigens found in gut extract, were inoculated into fully engorged *B. microplus* females (Toro-Ortiz et al., 1997). These antibodies decreased oviposition by 50 and 70 percent, respectively.

Production of Antibodies by Transgenic Plants

Production of mammalian antibodies by plants is a recently developed concept with significant potential. Applications of plant-produced antibodies include: disruption of development and action of plant pathogens and pests, antibody farming for therapeutic, affinity, or diagnostic uses, inhibition of plant regulatory factors, and processing of

environmental contaminants (Smith, 1996; Conrad and Fiedler, 1994; Hiatt, 1990; Hiatt and Mostov, 1993; Whitelam et al., 1994).

Hiatt et al. (1989) and During et al. (1990) demonstrated that transgenic tobacco could be engineered to produce functional mouse antibodies. Hiatt et al. (1989) transformed tobacco to express either the κ-light chain or the γ-heavy chain of the monoclonal 6D4 IgG_1. This antibody binds a low molecular weight phosphonate ester (P3). Complementary DNAs from a mouse hybridoma messenger RNA were incorporated into tobacco plants by *Agrobacterium*-mediated transformation. Plants expressing the κ-chain were crossed with those expressing a γ-chain yielding progeny that expressed both chains in the form of a fully functional IgG antibody. Antibody levels were found to be as high as 1.3 percent of total leaf protein.

During et al. (1990) engineered antibody producing tobacco by linking the κ- and γ-chain cDNAs in the same plasmid for transformation by *Agrobacterium*-mediated gene transfer. Fully functional assembled antibodies were detected in the lumen of the endoplasmic reticulum and in the thylakoid membrane of chloroplasts. Kappa- and γ-chains were found to assemble in the lumen of the endoplasmic reticulum. Tobacco plants have also been transformed to express other full-size antibodies (Hein et al., 1991; van Engelen et al., 1994; De Neve et al., 1993).

Ma et al. (1994) transformed tobacco to express IgG-like hybrid antibodies with either IgG or IgA heavy chains. This research was expanded with transformation of tobacco plants to express fully functional secretory IgA, consisting of κ-light chain, hybrid immunoglobulin α-γ heavy chain, murine joining chain, and rabbit secretory

component (Ma et al., 1995). Plants other than tobacco have also been transformed to produce whole functional antibodies (De Neve et al., 1993; Steiger et al., 1991).

Recombinant antibody fragments have also been expressed in transgenic plants (Firek et al., 1993). Recombinant antibody fractions are smaller than whole antibodies and require no post-transcriptional assembly, which may enable the fragments to access the target area more easily (Firek et al., 1993; Hein et al., 1991). Tobacco was transformed to express just the heavy chain variable region (H_v) of a monoclonal antibody at levels as high as 1% of the soluble protein fraction (Benvenuto et al., 1991). The single chain Fv fragment (scFv), consisting of the variable light chain and the variable heavy chain united by a flexible linkage, maintains the full antigen binding site and is the product of a single synthetic gene (Whitelam et al. 1994). Functional scFv proteins have been expressed in tobacco plants at levels of 0.06 to 0.1 % (Owen et al., 1992) and up to 0.5% of the total soluble protein fraction of leaf tissue (Firek et al., 1993). Monoclonal scFv proteins specific for the artichoke mottled crinkle virus (AMCV) virion expressed in tobacco plants (0.1% total soluble protein) specifically protected the plants from AMCV attack, reducing the incidence and symptoms of the disease (Tavladoraki et al., 1993).

Transgenic Insect Resistance

Much of the insect resistance found in modern crops is the result of traditional plant breeding programs, but an increasing proportion of this resistance results from genetic transformations (Gatehouse et al., 1992). Insect resistance factors introduced into transgenic lines usually utilize the resistance mechanism of antibiosis by causing a plant to produce a substance, generally a protein, that is toxic to the pest species (Lal and Lal,

1993). These toxic substances are often found in nature, and their genetic codes for production are determined (Boulter, 1993). To date, transgenic insect resistance genes have been introduced for expression of several types of proteins that are toxic to insects, including the *B. thuringiensis* insecticidal crystal proteins, proteinase inhibitors, enzymes, and plant lectins (Estruch et al., 1997; Gatehouse et al., 1992; Boulter, 1993; Lal and Lal, 1993).

Bacillus thuringiensis Insecticidal Crystal Proteins

The first insecticidal transgenic protein to be introduced into plants on a wide scale was the *B. thuringiensis* δ-endotoxin. This aerobic, spore forming, gram negative bacterium produces a crystalline endotoxin during sporulation (Raman and Altman, 1994). The species, *B. thuringiensis,* contains several subspecies which produce insecticidal crystal proteins with toxicity to different orders of insects. Crystalline proteins of subspecies *B. thuringiensis kurstaki* are highly and selectively toxic to members of the order Lepidoptera (Dulmage, 1981). Proteins of *B. thuringiensis israeliensis* were found to be highly toxic to larvae of blackflies and mosquitoes (Goldberg and Margalit, 1977). The most recently discovered subspecies, *B. thuringiensis tenebrionis,* produces crystalline proteins which display toxic effects against larvae of the order Coleoptera (Lal and Lal, 1993).

The insecticidal crystal proteins and the genes that direct their production have been grouped and named according to the order of insects to which the proteins are most toxic (Hofte and Whitely 1989). The *B. thuringiensis* proteins are referred to as Cry proteins and the genes as *cry* genes. There are fifteen known Cry proteins and corresponding *cry* genes. Specific modes of action have not been determined for each

Cry protein, but their toxic effects have been found to occur in the following generalized manner (Bauer, 1995). The Cry proteins are protoxins that must first be ingested by the target species, solubilized in the midgut, and proteolytically cleaved to activate the toxin (Ogiwara et al., 1992). The activated toxin binds to specific receptors on the midgut epithelium (Hoffman et al., 1988) and disrupts the plasma membrane (English and Slatin, 1992), which causes the cell to be subject to osmotic changes whereby they are lysed (Masson et al., 1995). The disruption of the midgut epithelium allows bacteria present in the gut lumen to move into the hemolymph, and the insect actually dies of a bacterial septicemia (Wilson and Benoit, 1990).

The various *cry* genes have been genetically engineered into a number of crop species to produce insect resistant transgenic plants. Successful introduction of a *B. thuringiensis* gene into plants was first accomplished by Vaeck et al. (1987) when tobacco plants were transformed to produce a *B. thuringiensis* toxin which was found to be sufficiently potent to kill first instar *Manduca sexta* (Linnaeus). Since that time *cry* genes have been introduced into cotton, with resultant toxic effects on pink bollworm (*Pectinophora gossypiella* (Saunders)), cotton leaf perforator (*Bucculatrix thurberiella* Busck), saltmarsh caterpillar (*Estigmene acarea* Drury), beet armyworm (*Spodoptera exigua* (Hübner)), and tobacco budworm (*Heliothis virescens* (Fabricius)) (Wilson et al., 1992, Mascarenhas et al., 1994, Perlak et al., 1990, Jenkins et al., 1993). Cry proteins expressed in tomato plants produced toxic effects on corn earworm (*Heliothis zea* (Boddie)), tobacco budworm (*H. virescens)*, tobacco hornworm *(M. sexta)*, and beet armyworm (*S. exigua)* (van der Salm et al., 1994 and Fischoff et al., 1987). Potato plants expressing Cry proteins were resistant to potato tuberworm (*Phthorimaea operculella*

(Zeller) (Jansens et al., 1995) and Colorado potato beetle (*Leptinotarsa decemlineata* (Say)) (Perlak et al., 1993, Arpaia and Ricchiuto, 1993, and Adang et al., 1993), and rice plants with *cry* genes were resistant to striped stemborer (*Chilo suppressalis* (Walker)) and rice leaffolder (*Nephotettix nigropictus* (Stal)) (Fujimoto et al., 1993). Many other plants have also been transformed to produce Cry proteins with resulting toxic effects on the insect pests that feed on them.

Other Insecticidal Proteins

Proteins other than *B. thuringiensis* Cry proteins exhibit insecticidal properties, and many have potential for production by transgenic plants (Estruch et al., 1997; Gatehouse et al., 1994. The first non-Bt protein gene to confer plant resistance to insects encoded for synthesis of the cowpea trypsin inhibitor (CpTI) (Hilder et al., 1987). In laboratory bioassays tobacco plants expressing the CpTI protein suffered only minor damage when fed upon by tobacco budworm in comparison to the non-CpTI expressing plants (Hilder et al., 1987). The larvae which fed on the CpTI producing tobacco plants either died or their growth and maturation were significantly hindered. Upon ingestion, CpTI protein acts to decrease the available pool of amino acids for uptake, thus reducing the nutrient supply needed for proper growth and development (Gatehouse and Boulter, 1983). The cowpea trypsin inhibitor gene in tobacco has detrimental effects on the survival of *H. zea* larvae (Hoffman et al., 1992).

Other proteinase inhibitors expressed in transgenic plants have conferred resistance to insects. The tomato and potato protease inhibitors, PI-II (trypsin inhibitor) and PPI-II (specific for chymotrypsin-like proteases), have both been shown to be deleterious to insects when expressed in transgenic tobacco (Johnson et al., 1989; Hilder

et al, 1993). Thomas et al. (1994) transformed alfalfa to produce an elastase inhibitor from *M. sexta* hemolymph. Feeding by thrips, *Frankliniella spp.*, was reduced on the transformed alfalfa in comparison to controls.

Other potential insecticidal proteinase inhibitors have been identified from their effects in an *in vitro* study by Rhabe et al. (1995). The Bowman-Birk trypsin/ chymotrypsin inhibitor (SBBI) had a significant negative impact on development and survival of pea aphids fed this protein in an artificial diet. The inhibitors beef pancreas trypsin inhibitor (BPTI) and potato carboxypeptidase (CPI) decreased pea aphid survival but had lesser effects on growth. In the same study other inhibitors, pepstatin and chymostatin also decreased survival and growth of pea aphids *in vitro* (Rhabe et al. 1995).

Enzymes are a potentially important group of insecticidal proteins. Although none have been expressed in transgenic plants, some enzymes have been shown to have insecticidal effects *in vitro*. Powell et al. (1993) fed soybean lipoxygenase type 1-B (LPO) in an artificial diet to third instar rice brown planthopper, *Nilaparvata lugens* Stal, and found that it greatly reduced survival. Two other enzyme classes were shown to have insecticidal activity against the green peach aphid (Purcell et al., 1994). Invertase and hexosyl-transferase obtained from fermentation broths were found to cause death of aphids fed these enzymes in an artificial diet. Upon further investigation, they found that these enzymes actually depleted the sucrose from the diet, and the aphids died of starvation. A promising new enzyme for insect control is chlolesterol oxidase (CO). *In vitro* studies of cholesterol oxidase fed to boll weevil larvae showed toxicity levels comparable to those of *B.thuringiensis* (Estruch et al., 1997).

Another group of proteins with insecticidal properties and potential for transgenic insect resistance are plant lectins (Estruch et al., 1997; Gatehouse et al., 1994). Many lectins have been found to exhibit insecticidal properties *in vitro*. Rhabe et al. (1995) screened thirty plant lectins incorporated into an artificial diet for toxicity to the pea aphid. Most were ineffective, but some caused significant mortality. The lectins with the highest aphicidal properties were: Concanavalin A (Con A), amaranth lectin (ACA), lentil lectin (LcH), and snowdrop lectin (GNA). Notably, all of the active lectins bind glucose/mannose or strictly mannose (GNA only). However, other mannose specific lectins tested were not toxic to aphids. Concanavalin A was also fed to several other species of aphids, *Aphis gossypii* Glover, *Aulocorthum solani* (Kalt.), *Macrosiphum euphorbiae* (Thomas), *Macrosiphum albifrons* (Essig), and *Myzus persicae* (Sulzer), as well as *A. pisum* (Rhabe et al., 1995). The species most affected by Con A, *A. pisum* and *M. albifrons,* were unable to reach adulthood before death. Other species were slightly affected, but not significantly.

Additional *in vitro* assays to test the effects of lectins on insects were performed by Powell et al. (1993). Rice brown planthopper nymphs were fed on artificial diet containing several individual plant lectins. Snowdrop lectin and wheat germ agglutinin (WGA) caused a significant decrease in planthopper survival when compared with the diet only control. Whereas Con A, horse gram agglutinin, jacalin agglutinin, potato lectin, and *Phaseolus vulgaris* agglutinin produced little or no effect on mortality.

Plants have been genetically transformed to express lectins with insecticidal properties. Tobacco plants expressing pea lectin were resistant to attack by larval *H. virescens* (Boulter et al., 1990). Pea lectin and cowpea trypsin inhibitor genes were both

successfully incorporated into tobacco plants by cross pollination of two transgenic lines, and their insect resistant effects were additive (Boulter et al., 1990). Most recently, the snowdrop lectin gene was incorporated into tobacco plants (Hilder et al., 1995). Aphids of the species *M. persicae*, reared on leaf discs and whole plants expressing snowdrop lectin, experienced a marked decrease in aphid population size and population growth rate. Snowdrop lectin has also been shown to be effective against the glasshouse potato aphid, *A. solani,* in laboratory bioassay and in transgenic potato (Down et al., 1996). For bioassay, snowdrop lectin was added to artificial diet at a concentration of 0.1 percent (w/v). Aphids fed the modified diet throughout the life cycle showed a 65 percent decrease in fecundity, and in another assay caused high mortality. Potato plants were transformed to express snowdrop lectin in a range of 0.3 to 0.4 percent of total soluble protein by *Agrobacterium*-mediated gene transfer and the cauliflower mosaic virus 35S promoter (CaMV 35S) (Gatehouse et al., 1996). Aphids fed on the transformed plants had a 60 percent decrease in fecundity versus those fed on control plants in growth chamber studies. In large scale glasshouse studies, control plants had aphid infestation levels four times greater than plants expressing snowdrop lectin (Down et al., 1996).

Overview of Plant Transformation Techniques

Progress made in genetics, recombinant DNA techniques, plant tissue culture, and methods for selection of transformed plant cells or tissues has greatly advanced the ability to improve crop plants by genetic transformation (Lal and Lal, 1993; Jenes et al., 1993). Plant transformation techniques include: *Agrobacterium*-mediated gene transfer, chemical-mediated gene transfer, biolistic or microprojectile bombardment, electroporation, micro- and macroinjection, and sonication of protoplasts. Not all

transformation strategies can be used successfully on all plant species. Preferred methods of gene transfer in dicotyledonous plants are *Agrobacterium*-mediated transfer and biolistic gene transfer (Jenes et al., (1993). For monocotyledonous plants, polyethylene glycol-mediated gene uptake, electroporation, and particle bombardment techniques are preferred (Jenes et al., 1993). A general description of each preferred method is given below.

Agrobacterium-mediated gene transfer (White, 1993) utilizes the tumor inducing bacterium, *Agrobacterium tumefaciens* (Smith and Townsend). Innate DNA transfer mechanisms are encoded in the virulence region of the tumor inducing Ti-plasmid of the bacterium. The virulence region encodes for excision and packaging of the transferred DNA, but virulence region genes are not transferred. Transferred DNA or T-DNA is also found on the Ti-plasmid in the T-DNA region. These genes do not encode any part of the transfer process. For genetic transformation, the T-DNA genes for tumorigenesis are disarmed, and desired genes are included in the T-DNA region of the Ti-vector. Plant tissue to be transformed is incubated with the modified bacteria, and then placed on selective media to retard growth of residual bacteria and kill nontransformed cells. This process is continued until surviving plants are regenerated and can be grown in soil.

In the process of biolistic gene transfer, DNA coated microprojectiles of gold or tungsten are introduced into intact plant cells or protoplasts by a microprojectile gun (Morrish et al., 1993). DNA for transfer is generally arranged as an expression cassette which includes a promoter sequence, a transcription initiation site, and a synthetic multi-linker followed by a polyadenylation signal. The multi-linker allows for insertion of reporter genes, genes for media selection, and the desired genes for transfer.

Polyethylene glycol mediated gene transfer (Lal and Lal, 1993; Jenes et al., 1993) is used to transform protoplasts. Protoplasts are plant cells without a cell wall. Polyethylene glycol increases the permeability of the cell membrane, and is used to stimulate protoplasts to uptake naked DNA. Transformed plants are regenerated from protoplasts in selective media.

According to the review by Lal and Lal (1993), electroporation is the process of applying high voltage to induce pore formation in protoplasts. Micro- or millisecond pulses of one thousand to two thousand volts are used to cause protoplasts to uptake DNA of various vector sizes. DNA concentrations are variable depending upon the species of plant to be transformed.

CHAPTER II

MATERIALS AND METHODS

Insects

Pea aphids used in this study were taken from a long-standing clonal colony at Oklahoma State University. Aphids were reared on broad bean plants, *Vicia faba* L., grown from seed in trays of potting soil in a closed room with a light: dark cycle of 16:8 hours at 26 °C. In order to maintain a steady supply of young plants for the aphids to feed on, older plants were removed as younger plants emerged from the soil. Aphids were transferred from older bean plants to younger plants by brushing them from the older plants.

Aphid Dissections

Apterous fourth instars and adult pea aphids were obtained from the colony by shaking the aphids from the plants on to a wire sieve which held the larger aphids and allowed the smaller aphids to pass through. Large aphids were held on ice in a petri dish to limit mobility. These aphids were dissected in homogenization buffer (0.15 M phosphate-buffered saline, pH 7.2, (PBS) containing proteinase inhibitors: leupeptin (1μM), 4 - (2 - aminoethyl) - benzenesulfonylfluoride (AEBSF) (0.42 mM) and EDTA (disodium salt) (1 mM)). Each aphid was submerged in homogenization buffer with fine forceps, and the midgut was exposed by piercing the pronotum with a #2 insect pin. The midgut was then removed from the aphid with fine forceps and placed into

homogenization buffer in a nonsterile 1.5 ml microcentrifuge tube held on ice. Midguts collected on the same day were placed in the same tube. Midguts were stored at -20 °C until needed.

Aphid Midgut Samples

Pea aphid midguts were disrupted by sonication using the Vibracell VC50 probe sonicator (Sonics and Materials, Inc., Danbury, CT) for two 30 second pulses at setting 20. Samples were chilled in an ice bath between pulses to prevent protein degradation due to heating during sonication. Protein content of sonicated midgut was determined as described below, and the sonicate was used for rabbit immunizations, analytical sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE), immunoblotting, and enzyme linked immunosorbant assay (ELISA) as described below.

Protein Assay

Measurement of protein content of midgut homogenates and IgG preparations was accomplished using the bicinchoninic acid protein assay (Smith et al. 1985). Two hundred fifty microliters of reagent were placed in each well of a non-sterile, 96 well, flat-bottomed, polystyrene, tissue culture plate (Corning, Corning, NY), using a step pipetter (Eppendorf Repeater 4780, Brinkman Instruments, Westbury, NY). Bovine serum albumin (BSA) standards were prepared at concentrations of 1.0, 2.0, and 3.0 μg of protein in 20 μl of the preferred blanking agent (PBS for IgG preparation samples or homogenization buffer for aphid gut samples).

Samples to be assayed were serially diluted. Twenty microliters of the blanking agent, BSA standards, or sample dilutions were each assayed in triplicate. Each plate was then slowly agitated for two to three minutes before incubation at 60 °C for 30 minutes. Optical densities at 562 nm were determined using a microtiter plate spectrophotometer (Bio-tek Instruments, Winooski, VT). For the three wells of each standard or sample, average optical densities were calculated, and linear regression analysis was performed to calculate the amount of sample protein per well. Protein concentrations were used to determine the appropriate volume of midgut homogenate to use in SDS-PAGE, immunoblots, ELISA, and rabbit immunizations; or purified IgG samples to be incorporated into aphid diet.

Immunizations

Midgut sonicates were adjusted to a final concentration of 1 mg/ml protein in homogenization buffer. One ml of midgut sonicate in buffer was emulsified with 1 ml of Freund's Complete Adjuvant. Each rabbit was injected with 1 mg protein in 2 ml adjuvant-protein emulsion divided evenly between two intramuscular sites in the hind legs and two subcutaneous sites near the neck.

Rabbits were boosted with a 1:1 emulsion of Freund's Incomplete Adjuvant and aphid gut sonicate (0.5 mg protein) at 14 and 28 days post-initial immunization and thereafter as needed. Each boost was divided between four injection sites as described above. Rabbits J1 and J2 received the initial immunization and three booster injections. Rabbits A2, A3, and B2 received an initial immunization and four booster injections.

Collection of Blood and Preparation of Sera

Blood was collected from an ear vein of each rabbit before initial immunization and seven to ten days after each boost. Blood samples were held for 12 hours at 4 °C and then centrifuged at 1000 x g, for 20 min (10 °C) to separate serum. Serum was removed, divided into 0.5, 1.0, and 2.0 ml aliquots in plastic cryovials, and stored at -20 °C for later use in immunoblotting, ELISA, DEAE batch purification of IgG, and incorporation into artificial diets.

Analytical Sodium/Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

Proteins from midgut samples were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli. (1970). Resolving gels containing 12% acrylamide were prepared by mixing 5 ml of 1.5 M Tris-HCl, pH 8.8 (18.2 g Tris base in 80 ml distilled water, pH adjusted to 8.8 with 1-4 N HCl, final volume brought to 100 ml with distilled water), 7.9 ml deionized water, and 8ml of acrylamide-bis (30 % T, 2.7 % C) in a vacuum flask. The flask was connected to a faucet aspirator and placed in a water bath sonicator to remove gas. When gas was no longer liberated, one hundred microliters of 10% ammonium persulfate and 10 µl of N,N,N′,N′-Tetra-methyl-ethylenediamine (TEMED) (Biorad, Hercules, CA) were added to the mixture. Resolving gels were then poured into packaged pre-made mini-gel (8 by 10 cm) cartridges (Jule, Inc., New Haven, CT), overlaid with deionized water, and allowed to polymerize. Stacking gel was prepared by mixing 5 ml of 0.5 M Tris HCl, pH 6.8 (consisting of 6.1 g Tris base in 80 ml of deionized water, pH adjusted to 6.8 with 0.25 N HCl, and the final volume brought to 100 ml with deionized water) with 12.2 ml

of deionized water, and 2.7 ml of acrylamide-bis (30% T and 2.67% C). The mixture was degassed, and 100µl of 10% ammonium persulfate and 20 µl of TEMED were added to the mixture and dispersed by gentle swirling. Stacking gels were then poured on top of the resolving gels, combs were inserted, and the stacking gels were allowed to polymerize.

Midgut samples for electrophoresis were added in equal volume with 1X sample buffer containing 2-mercaptoethanol (4.8 ml of deionized water, 1.2 ml of 0.5 M Tris-HCl pH 6.8, 2 ml 10% SDS, 1.0 ml glycerol, 0.5 ml of 0.05% bromophenol blue, and 0.5 ml of 2-mercaptoethanol). Samples were boiled in a water bath for 5 minutes prior to loading of gels. Three to five microliters of wide range molecular weight markers (Sigma, St. Louis, MO) were loaded into a sample lane of each gel so that molecular weights of sample proteins could be estimated. Fifty to 100 micrograms of protein were added to trough gels for preparation of an electrophoretogram to be electroeluted for use in immunoblotting. Gels were run for 45 minutes at 200 volts constant current using a mini Protean II dual slab gel system (Biorad, Hercules, CA). Gels were then stained with Coomassie Blue R-250 (Laemmli, 1970) or silver stained (Morrissey, 1981). Gels for immunoblots were stained with Rapid Coomassie stain (Research Products International Corp., Mount Prospect, IL) until the molecular weight markers were just visible (Thompson and Larson, 1992).

Immunoblotting

Immunoblotting was used to determine the specificities of antibodies generated by immunized rabbits and the molecular weights of proteins/polypeptides with which those

antibodies bound (Towbin et al., 1979; Thompson and Larson, 1992). Immunoblotting was performed with sera from two groups of rabbits. Group 1 consisted of rabbits C, J1, and J2. Group 2 rabbits were A2, A3, and B2. Aphid midgut proteins (75 μg protein per trough for Group 1 blot and 50 μg protein per trough for the Group 2 blot) separated by SDS-PAGE were stained with Coomassie blue R-250. The proteins were electrophoretically transferred to nitrocellulose membrane at 100 volts for one hour using Mini Trans-blot Electrophoretic Transfer Cell (Biorad, Hercules, CA). Once the stained proteins were transferred, the nitrocellulose sheet was cut into strips and incubated in Tris-saline buffer (10mM Tris-HCl, 0.9% NaCl, pH 7.4) containing 5% non-fat dry milk, Bovine Lacto Transfer Technique optimizer (BLOTTO), for 45 minutes (Johnson, et al., 1983).

The BLOTTO was removed, and the strips were incubated with gentle agitation for one to two hours in appropriate dilutions of rabbit sera. Sera used were from a non-immunized rabbit (C) and two immunized rabbits (J1 and J2), for the Group 1 blot. Each serum was diluted to 1:10 and 1:25 in BLOTTO for this blot. Pre- and post-immunization sera of rabbits A2, A3, and B2 were used in the Group 2 blots. Each of these sera were diluted to 1:50 with BLOTTO, and then reacted with nitrocellulose strips bearing the electroeluted electrophoretogram.

After the first antibody reaction, nitrocellulose strips were washed two times with BLOTTO and three times with BLOTTO containing 0.05% Tween-20 for 5 minutes each. The strips were incubated for 60 minutes in goat anti-rabbit IgG horseradish peroxidase conjugate, diluted 1:200 in BLOTTO. Immunoblots were washed again in BLOTTO and BLOTTO containing Tween-20. Immunoblots were developed by

incubating strips in a solution consisting of 60 ml of Tris-saline buffer, 30µl of 30% hydrogen peroxide and 12ml of 4-chloro-1-naphthol (Sigma) at 3mg/ml in methanol. Reactions were stopped by removal of development solution and by rinsing nitrocellulose strips with Tris-saline buffer.

Densitometry

Immunoblots of post-immune sera of rabbits A2, A3, and B2 were compared using densitometry (UA-5 Absorbance Detector, ISCO, Lincoln, NE). Reflected light from the blots was read at a rate of 5mm/min. Densitometry tracings of the wide range molecular weight markers were used to estimate the size of the proteins where peaks occurred on the tracings of A2, A3, and B2 sera reacting with separated pea aphid proteins/ polypeptides. Densitometry tracings of the three rabbits were compared for differences in number and magnitude of peaks.

Enzyme-linked Immunosorbant Assay

Immulon® 2 surface treated, flat bottom, polystyrene microtitre plates (Dynatech Laboratories, Chantilly, VA) were coated with 500 ng of aphid midgut protein in 100 µl per well of 0.05 M carbonate-bicarbonate coating buffer, pH 9.6 (anhydrous Na_2CO_3 (1.59g) and $NaHCO_3$ (2.93 g) in one liter of distilled water). Plates were sealed with acetate tape, incubated at 37 °C for 60 minutes, and stored overnight at 4 °C. Coated plates were washed three times for two minutes each with 0.15 M phosphate buffered saline, pH 7.2, containing 0.05 % Tween-20. The first antibody, pre- or post-immunization rabbit sera from rabbits A2, A3, or B2, were serially diluted from 1:20 to

1:40,960 in PBS with Tween-20 and incubated for one hour. The wells were washed three times for two minutes each in PBS with Tween 20 for two minutes each wash. One hundred microliters of the second antibody, consisting of a 1:5,000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase, were added to each well and incubated for one hour at room temperature. The wells were washed as described above. One hundred microliters of substrate consisting of O-phenylenediamine (10.0 g) in 25 ml of 0.1 M citrate-phosphate buffer, pH 5.0 (50:50 mixture of 0.1 M disodium hydrogen phosphate and 0.1 M citric acid) with 110 µl of 30% hydrogen peroxide were added to each well. The plate was then incubated in the dark for 30 minutes. Development was stopped by the addition of 50 µl of 0.5 M sulfuric acid (H_2SO_4). Absorbance was measured at 490 nm by microplate spectrophotometer.

Batch Preparation of Rabbit γ-Globulin Using DEAE-Sephadex

The DEAE-sephadex batch technique of Rief (1969) was used to purify and concentrate IgG from sera of rabbits C, J1, and J2. Phosphate buffer (0.01M), pH 6.5 was prepared by dissolving 3.864 g of KH_2PO_4 (mw 136.09) and 2.020 g of K_2HPO_4 (mw174.18) in 4 liters of deionized water.

To prepare the gel matrix, 10 g of DEAE-sephadex were gently stirred for 30 minutes in 1200-1400 ml of 0.01 M phosphate buffer. After the gel had swollen (4-5 hr.) excess buffer was poured off. The gel was then resuspended in 800 mls of phosphate buffer and stirred slowly for 15 min. Gel was then trapped on a 12.5 cm diameter Qualitative P8 filter paper circle (Fisher Scientific, Pittsburgh, PA) in a Buchner funnel

using mild suction, washed slowly with 1 L of 0.01 M phosphate buffer, transferred to a beaker, and stored covered at 4 °C.

To separate the IgG, 25 ml of rabbit serum were added to 40 g of moist gel and allowed to stand for 1 hr at 4 °C with occasional mixing. Gel-serum mixture was then filtered and washed slowly with 100 ml of phosphate buffer. Forty grams of moist gel were added to the filtrate and allowed to stand for 1 hr at 4 °C with occasional mixing. The mixture was filtered and washed again with 100 ml of 0.01 M phosphate buffer. The filtrate was concentrated to approximately 25 ml in standard cellulose dialysis tubing (12,000 to 14,000 mw cutoff, Spectrum Medical Industries, Inc., Los Angeles, CA) against Aquacide II, extremely high viscosity sodium salt of carboxymethyl-cellulose (Calbiochem Corporation, La Jolla, CA). Forty grams of moist gel were added to the concentrated filtrate and stirred for 1 hr at 4 °C. The mixture was filtered and washed with 100 ml 0.01 M phosphate buffer. The filtrate was concentrated again to 25 mls, mixed with gel, and filtered and washed as described above. The filtrate was then adjusted to pH 7.5 with 1.0 M K_2HPO_4 and reconcentrated to less than 10 ml. IgG preparations were then aliquotted in 0.5 ml portions and stored at -20 °C.

Purity of IgG Preparation

Purified IgG isolated from the sera of three rabbit were evaluated by double immunodiffusion (DID) and by immunoelectrophoresis (IEP). For DID, sheets of GelBond (FMC Corp., Rockland, ME) were sealed (hydrophobic side toward glass) with water to 7.5 cm X 5.0 cm glass slides. Glass chips (0.1 cm high) were placed on each corner, and another slide was placed on the chips to complete the sandwich assembly.

The assembly was then placed on a slide warmer. Agar gel was prepared by boiling 30 mg agarose (Sigma, Type IV, Special High EEO, St. Louis, MO) in 5 ml of Tris-barbital buffer (High Resolution Buffer, Gelman, Ann Arbor, MI). Warm gel was added between the top and bottom of the slide assembly with a Pasteur pipette and allowed to cool until solidified. Upon solidification, 4 mm wells were punched into the gel, and gel and liquid were removed with a Pasteur pipette attached to a vacuum. Four microliters of IgG preparation (dilutions of 1:1, 1:5, 1:25, 1:125, 1:625, or 1:3125) were added to each of the six outer wells of each gel. In the center well, 4 µl of goat anti-rabbit IgG were added. Gels were incubated overnight in a moist chamber to allow substrates to diffuse and immune complexes to form. Unprecipitated protein was removed by alternating steps of pressing the gels for 10 min and washing the gels for 15 minutes in 0.2 M NaCl. After the fourth press, gels were washed twice in water for 10 min. each, pressed, dried, and stained with Coomassie Blue R-250. Double immunodiffusion gels were examined for precipitin lines at each concentration for each rabbit IgG preparation.

For immunoelectrophoresis, 0.1M Veronal Buffer was made by adding 0.1 M HCl to 0.1 M sodium barbital (30.9 g in 1.5 L distilled water) until the pH was 8.2. Buffer was then stored at 4 °C until used.

Agarose gel was prepared by boiling 1.5 g agarose (Type IV, Special High EEO, Sigma, St. Louis, MO) in 100 ml of Veronal buffer. One ml of 1% Thiomersol was added to prevent mold growth. Gel was applied with a 10 ml pipette to frosted end slides placed on a slide warmer. Gels were allowed to solidify for 30 minutes at room temperature and stored at 4 °C until used. A 4 mm well and a 6.5 cm by 4 mm trough were punched into each gel, and gel and liquid were removed by suction.

Six microliters of each IgG preparation were added to the 4mm well of each gel and placed on a flatbed electrophoresis unit with circulating cooling. The buffer chamber was filled with 750 ml of Veronal buffer, and buffer saturated microporous wicks were placed with one end on the agar layer and one end in the buffer to complete the circuit. Electrophoresis was performed at 20 milliamps for 2 hours.

Forty microliters of goat anti-whole rabbit serum were then added to the trough in each gel, and gels were allowed to incubate in a moist chamber for 36 hours at room temperature (19 °C). To remove unprecipitated protein, gels were washed in several changes of 0.9% saline for 24 hr, and then in distilled water for 12 hr. Gels were dried at 37 °C overnight with a piece of moist filter paper on the surface of the gel. After drying, gels were stained with 0.5 g/L Buffalo black in staining solvent consisting of 2000 ml dH_2O, 160 ml 95% ETOH, and 40 ml glacial acetic acid, destained in staining solvent, and dried at room temperature. Stained precipitin arcs for each rabbit IgG preparation were then viewed and compared to published standards to determine presence of IgG and impurities.

Artificial Diet Preparation

Diet Comparisons

The artificial diets chosen for evaluation in these studies were those of Akey and Beck (1972), Febvay et al. (1992), and Abisgold et al. (1994). Diets were prepared with modifications as described below. Diets differed mainly in amino acid composition and quantities of amino acids and sucrose, but were similar in regard to vitamins, minerals, trace metals, and other inorganic nutrients. Diets were prepared using the non-amino

acid or sucrose nutrients as reported by Akey and Beck (1972), except that riboflavin content was reduced to 0.5 mg per 100 ml in the diets of Febvay, et al. (1992) and Abisgold, et al. (1994). The amounts of amino acids and sucrose per 100 ml of each diet are provided in Table 1. Other nutrients based on weight per 100 ml of diet were as follows: K_3PO_4 (500 mg), $MgCl_2 \bullet 6H_2O$ (2.5 mg), Salt Mix #2 (5 mg), $MgSO_4 \bullet 7H_2O$ (242 mg). Trace metals in the form of chloride salts were added as follows: Cu^{2+} (120 μg), Fe^{2+} (920 μg), Mn^{2+} (220μg), Na^{2+} (1000μg), and Zn^{2+} (400μg). Vitamins were added to the diet medium at the following weights per 100 ml of diet: ascorbic acid (100 mg), biotin (0.1 mg), calcium pantothenate (5 mg), choline chloride (50 mg), folic acid (1 mg), I-inositol (meso) (50 mg), nicotinic acid (10 mg), p-aminobenzoic acid (10 mg), pyridoxine HCl (2.5 mg), thiamine HCl (2.5 mg), and riboflavin (5 mg). Diet components were dissolved in two times distilled water and adjusted to pH 7.6. Water was added to produce a final volume of 100 ml. Diets were divided into 10 ml aliquots in sterile plastic screw-top tubes and stored at -20 °C.

Base Diet Preparation

The artificial diet of Febvay et al. (1992) was chosen as the base diet for testing the effects of serum or purified antibody. For the base diet, a 1.5 X diet solution was prepared by dissolving components in a final volume of 66 ml. This allowed as much as one third of the volume fed to aphids to be an addition of serum while maintaining the same nutrient composition in all treatments.

TABLE 1.

AMINO ACID AND SUCROSE CONTENT OF THE THREE ARTIFICIAL DIETS.

	Akey and Beck, 1972	Febvay et al., 1992	Abisgold et al., 1994
Amino Acids (mg/100mL)			
Alanine	100.0	179.0	25.4
β-Alanine	---	6.2	---
β-Alanyl Tyrosine	---	94.6	---
Arginine	400.0	245.0	124.1
Asparagine	300.0	298.0	94.1
Aspartate	100.0	88.0	94.1
Cysteine	50.0	30.0	16.4
Cystine	5.0	---	---
GABA	20.0	---	---
Glutamate	200.0	149.0	61.8
Glutamine	600.0	446.0	120.6
Glycine	20.0	167.0	4.5
Histidine	200.0	136.0	67.5
DL Homoserine	800.0	---	---
Isoleucine	200.0	165.0	57.1
Leucine	200.0	232.0	57.1
Lysine	200.0	351.0	63.6
Methionine	100.0	72.0	21.3
Ornithine	---	9.0	---
Phenylalanine	100.0	232.0	23.5
Proline	100.0	129.0	32.8
Serine	100.0	124.0	30.0
Threonine	200.0	127.0	51.8
Tryptophan	100.0	43.0	29.1
Tyrosine	20.0	39.0	5.4
Valine	200.0	191.0	51.0
Sucrose (g/100mL)	35.0	20.0	20.5

Diet Sachet Preparation

Diet sachets were prepared using a 4 cm by 4 cm square of Parafilm 'M' (American National Can, Greenwich, CT) stretched over one end of a fire-polished glass ring (2.54 cm diameter by 2.54 cm height). Parafilm covered glass rings were transferred to a biological safety cabinet (Nuaire, Plymouth, MN) and surface sterilized under ultraviolet light (40 microwatts per square cm) for one to two hours. One fourth squares (2 cm by 2 cm) of Parafilm were sterilized in 70 percent ethanol for one to two minutes and dried on a test tube rack in a biological safety cabinet in sterile air flow. The appropriate volumes of serum or IgG and/or distilled water were added to the diet as needed, and then each mixture was passed through a 0.22 micron Millipore GV-4 membrane filter (Millipore Products, Bedford, MA) into a sterile screw-top plastic test tube.

A droplet of the diet preparation (200 µl) was placed on the top of each parafilm covered glass ring. A dried quarter square of parafilm was stretched almost to the breaking point and carefully pulled down over the drop of diet so that it spread out but did not spill over the edge. The two parafilm layers were then pressed together around the edge of the glass ring to form a tight seal around the diet. The above procedures were performed under sterile conditions. After the sachets were assembled, they were labeled and stored on trays at -20 °C. Leaky or damaged sachets were considered to be contaminated, and they were discarded.

Aphid Cages and Feeding Platforms

Cages were constructed to fit snugly down over the edge of the diet sachet to confine aphids, and feeding platforms were used to stabilize and organize the diet sachets during feeding trials. Cages were made by cutting out the end of a #9 hollow core stopper (Nalgene, Rochester, NY) with a scalpel and covering the narrow end with a piece of fine mesh cloth secured by hot glue. Excess glue and cloth were trimmed using scissors so that the cages could be stacked when not in use. Feeding platforms were constructed of a flat wooden board with rows of 1.27 cm (1/2 inch) high sections of 1.91 cm (3/4 inch) wooden dowel secured to the board with hot glue. Pegs were set at a spacing of approximately 3.81 cm (1.5 inches).

Collection of Nymphs and Handling of Aphids

Newborn nymphs were used for all feeding trials. They were collected before each trial as follows: Adult aphids were collected by placing aphids in a sieve with 1.70 mm openings in the mesh and by shaking the sieve to release smaller aphids. The larger aphids were placed in a petri dish and any remaining nymphs were removed. Nymphs were identified by the lack of an elongated pygidium. A preliminary study revealed that this morphological feature was present after the final molt. One adult aphid for every two nymphs needed for the feeding trial was placed on caged broad bean plants in the growth chamber at 21 °C. Adult aphids were allowed to feed and produce offspring for 24 hours before each feeding trial. After 24 hours adult aphids and first instars were removed from plants by breathing on them to cause them to drop from the plant without harm. Adult aphids were returned to the colony, and the first instars were transferred to aphid

cages with a fine tipped paintbrush (moistened with tap water). Feeding sachets were then fitted into the open mouths of the cages, turned over, and tapped lightly to drop the nymphs onto the surface of the sachet where they could pierce the thin layer of parafilm and imbibe the diet preparation. Caged sachets were then placed over the pegs on a feeding platform.

The caged sachets were turned over and gently tapped to drop the aphids from the sachet surface to the cloth of the cage top so that aphids would not be lost during data collection. The feeding sachet was removed from the cage top. Aphids were counted and newly produced nymphs were removed while being viewed under the dissection scope. Fresh feeding sachets were introduced at this time, if required. Sachets were again placed into the open mouth of the cage, and aphids were tapped back onto the feeding surface.

Containment of Aphids on Excised Leaves and Whole Plants

Explants were prepared by removing a leaf from a bean plant from near the stem, leaving a large length of the petiole. The leaf petiole was wrapped tightly in cotton and inserted into a water-filled microcentrifuge tube from which the lid was removed. The explants were then laid in plastic petri plates. Any excess water was wicked up using small pieces of paper towel so that the first instars would not be trapped in a water droplet. Aphids were then placed on the explants to feed. When needed, aphids were removed from the explants by breathing on them.

Aphids were confined on leaves of intact plants by means of a cage that fit around a single leaf. A cage consisted of a plastic snap-shut push-pin box with a clothespin attached to one face. The box had vents covered with fine mesh cloth for air flow, and a

small indentation melted into the top edge of one end of the box such that when the box was closed the leaf petiole could rest in the indention and not be crushed. The clothespin attached to the cage was used to affix the cage to a ring-stand so that the cage would not have to be supported by the plant. Again, when needed, aphids were removed from the leaf and captured in the box for examination by breathing on them.

Feeding Trials: Experimental Design

Feeding trials were designed to measure the effects that specific treatments would have on aphid survival, developmental time, and fecundity. Groups of aphids were placed on feeding sachets of a particular treatment. A single group of aphids on a feeding sachet of a particular treatment was considered to be a replicate. Living aphids, adults, and offspring produced were counted each day. Methods of data collection and specific details of each feeding trial are explained below.

Data Collection

Data were collected every day for each replicate of each treatment by viewing aphids of each sachet under a dissection microscope. Survival data were obtained by counting the number of the original aphids still living on each sachet. Dead aphids were discarded. The number of adult aphids was determined by counting the number of surviving aphids possessing an elongated pygidium. The number of newborn nymphs was recorded, and they were then discarded.

Sachet Changing Frequency

An experiment was performed to determine if handling practices affected aphid survival, development, and fecundity. Groups of twenty first instars were fed on the artificial diet of Febvay et al. (1992) provided in feeding sachets at room temperature (approximately 19 °C). Three replicates of each of four treatments were conducted. Treatments were time intervals for changing sachets. The sachets of treatment A were changed weekly, treatment C sachets were changed daily, and the sachets of treatments B and D were changed every other day. In order to find if an extra day on the plant would increase survival, the nymphs in group D were allowed to feed on the plant until they were 24 to 48 hours old before being transferred to the diet. All replicates were conducted simultaneously to minimize variation. Data were collected over a period of 40 days as described above.

Diet Comparison

This study was designed to compare survival, development, and nymph production of aphids raised on whole plants, excised leaves (explants), and three artificial diets. The diet that best supported survival, shortest developmental time, and greatest fecundity was to be chosen for the base diet for future experiments. Groups of twenty first instars were placed on sachets of each of the artificial diets of Febvay et al. (1992), Abisgold et al. (1994), or Akey and Beck (1972) as described earlier; on explants; or on caged bean leaves and allowed to feed over a period of 26 days. Four replicates of each treatment were conducted simultaneously in a growth chamber with a light: dark cycle of 14: 10 hours at temperature 21 ± 1 °C and relative humidity of 70%. Diet sachets and explants were changed every other day. Surviving aphids, adult aphids, and newly

produced nymphs were counted and recorded daily for each sachet, explant, and caged leaf.

Effects of Pooled Pre-Immunization Rabbit Sera

This experiment was designed to compare the effects of different dilutions of pre-immunization rabbit serum in artificial diet on aphid survival, development, and production of nymphs. Rabbit serum, pooled from pre-immunization reserves of rabbits C, J1, and J2, was added to the 1.5X base diet to give final serum dilutions of 1:3, 1:6, 1:15, and 1:30 in complete diet. A group of twenty first instars was raised on a sachet of each of the four serum dilutions and diet only for 26 days. Four replicates of the five treatments were conducted at the same time in a growth chamber with a light: dark cycle of 14: 10 hours at temperature 21 ± 1 °C and relative humidity of 70%. Sachets were changed every other day. Surviving aphids, adult aphids, and newly produced nymphs were counted and recorded daily for each sachet. Data were collected as previously described above.

Comparisons of Individual Pre-Immunization Rabbit Sera and Post-Immunization Sera of Rabbits J1 and J2

This experiment was designed to and to compare the effects of two dilutions of pre- and post-immunization serum of the rabbits C, J1, and J2 on aphid survival, development, and production of nymphs. Treatments consisted of diet only, diet containing pre-immunization serum of rabbits C, J1, or J2 at dilutions of 1:15 or 1:30, and diet containing post-immunization serum of rabbits J1 or J2 at dilutions of 1:15 or 1:30. Groups of twenty first instars were reared on sachets containing one of each of the twelve treatments. Four replicates of each treatment were conducted simultaneously in a

growth chamber with a light: dark cycle of 14: 10 hours, temperature 21 ± 1 °C, and relative humidity of 70%. Sachets were changed at two day intervals. Due to the limited supply of pre-immunization sera, the duration of the experiment was just 12 days. Data were collected as previously described.

This experiment was performed again with higher dilutions of sera (1:15, 1:10, and 1:5) for one week to determine the effects of these higher serum dilutions. Only data on survival were recorded. All other methods were as previously described.

Screening of Sera

Due to differing aphid survival rates on diet containing sera of individual rabbits, this study was performed to determine which rabbits had sera with low toxicity to aphids and could be used for future study. Sera from five rabbits, A2, A3, B1, B2, and B3, were tested for toxic effects on pea aphids over a week-long period. Groups of twenty first instars were reared on each of the following treatments: diet only or sera from the following rabbits: A2, A3, B1, B2, and B3 incorporated into the base diet at dilutions of 1:5, 1:15, and 1:30. Three replicates were conducted of each treatment. All replicates were conducted at the same time in a growth chamber with a light: dark cycle of 14: 10 hours at temperature 21 ± 1 °C and relative humidity of 70%. Numbers of surviving aphids were recorded daily for each sachet.

Comparison of Pre- and Post-Immunization Sera of A2, A3, and B2

This experiment was conducted to compare effects of pre- and post-immunization sera of rabbits A2, A3, and B2 on aphid survival, development, and nymphal production. Pre- and post-immunization sera of rabbits A2, A3, and B2 were fed to aphids at dilutions of 1:9, 1:15, and 1:30 in the base diet. Groups of twenty first instars were placed on

sachets of each dilution of each rabbit serum and on diet only. Four replicates of each treatment were conducted simultaneously in a growth chamber with a light: dark cycle of 14: 10 hours at temperature 21 ± 1 °C and relative humidity of 70%. Surviving aphids, adult aphids, and newly produced nymphs were counted and recorded daily for each sachet.

Comparison of Purified IgG and Whole Serum

This experiment was conducted to compare the effects on aphid survival, development, and nymph production produced by incorporation of purified IgG and whole sera of non-immunized (C) and immunized rabbits (J1 and J2) into artificial diet. Whole sera of rabbits C, J1, and J2 were added to the base diet at dilutions of 1:9, 1:15, and 1:30. Protein content of each purified IgG preparation was determined using the bicinchoninic acid protein assay (Smith et al. 1985). The IgG preparations were added to the diet based upon the knowledge that IgG is found between the levels 8 and 16 mg of protein per milliliter of rabbit serum (Harlow and Lane, 1988). For the 1:9 dilution of IgG preparation, 1.33 mg of protein were incorporated into 1 ml of the base diet and water added. The protein contents of the dilutions of 1:15 and 1:30 of the IgG preparations were respectively 0.8 mg and 0.4 mg per ml of total diet. A group of twenty first instarswas placed on a sachet of each dilution of each rabbit serum or IgG preparation. Four replicates of each treatment were conducted at the same time in a growth chamber with a light: dark cycle of 14: 10 hours at temperature 21 ± 1 °C and relative humidity 70%. Surviving aphids, adult aphids, and newly produced nymphs were counted and recorded for each sachet as previously described.

Data Analysis

Survival data was recorded by counting the living aphids on each sachet. The percent survival for each day for each sachet (replicate) was calculated by dividing the number of surviving aphids by the starting number of aphids. The percent survival data of each replicate of a treatment were averaged for each day to give the mean percent survival for each treatment on each day. Developmental data were calculated by dividing the number of adult aphids by the number of surviving aphids on each sachet for each day to give the percentage of surviving aphids that were adults. The percent adult data for the replicates of each treatment were averaged for each day to give the mean percentage of surviving aphids which were adults.

The number of nymphs produced per surviving adult aphid was calculated by dividing the recorded number of nymphs produced on a sachet on a day by the number of live adult aphids present on that sachet on that day. At the end of the study the nymphs produced per adult per day were summed for each replicate to give the total (cumulative) nymphs produced per adult over the length of the study for that particular replicate. The total nymphs produced per adult for each replicate were then averaged to give the mean total nymphs produced per adult for each treatment.

Due to the bulk of data recorded, only survival and developmental data from certain days of a study were analyzed and reported. Survival data was analyzed at equal intervals in order to portray the data accurately. Developmental data was analyzed on the day that the mean percentage of surviving aphids that were adults of one of the treatments was greater than 80 or 90 percent.

Diet comparison and comparison of pre-immunization sera data were analyzed using analysis of variance ($P < 0.05$). If one or more means were determined to be different, then Newman-Keuls test of means comparison ($P < 0.05$) was used to determine which means were different. Comparisons of pre- versus post-immunization sera and whole sera versus purified IgG preparations data were compared using Student's T-test ($P < 0.05$).

CHAPTER III

RESULTS

Feeding Trial Comparing Diet Sachet Changing Frequencies

Aphids were reared on the Febvay diet with sachet changes once per week (treatment A), once a day (treatment C), or once every other day (treatments B and D). Aphids subjected to treatment D were allowed to feed on the plant for an extra day before being transferred to the diet. On the sixth day of the experiment aphid survival for all treatments was greater than 80% (Table 2). Analysis of variance revealed that aphid survival was not significantly different for any of the four treatments at days 6, 13, 20, or 27. Aphids which were supplied a fresh sachet weekly had a notable reduction in survival from day 13 (83.2 % surviving) through day 20 (49.5 %). Analysis of variance with Newman-Keuls test showed aphid survival on Treatment B (sachet changed every other day) to be significantly greater than treatments A, C, and D at day 34. By day 40 all treatments had mean survival of less than ten percent.

Aphids began molting to the adult stage on day six and all surviving aphids had become adults by day 24 in all treatments (Table 3). On day 19 greater than 90% of the surviving aphids on at least one treatment had molted to the adult stage. Comparisons of mean percentages by ANOVA of surviving aphids which had molted to the adult stage by Day 19 revealed no significant differences among developmental rates for the four treatment groups.

TABLE 2.

MEAN PERCENT SURVIVAL ± S.D.[†] OF PEA APHIDS REARED ON THE FEBVAY DIET WITH DIET SACHET CHANGES EVERY DAY, EVERY OTHER DAY, OR WEEKLY.

Treatment	Frequency of Sachet Change	Day 6	Day 13	Day 20	Day 27	Day 34	Day 40
Febvay A	Weekly	89.8 ± 5.0	83.2 ± 7.4	49.5 ± 19.2	39.1 ± 16.6	18.6 ± 15.2^{A}	1.7 ± 2.9
Febvay B	Every 2 Days	95.0 ± 5.0	85.0 ± 8.7	75.0 ± 10.0	68.3 ± 7.6	43.3 ± 2.9^{B}	10.0 ± 10.0
Febvay C	Every Day	86.7 ± 11.5	78.3 ± 10.4	75.0 ± 8.7	61.7 ± 12.6	13.3 ± 5.8^{A}	0.0 ± 0.0
Febvay D*	Every 2 Days	83.3 ± 2.9	76.7 ± 2.9	68.3 ± 10.4	61.7 ± 20.2	20.0 ± 13.2^{A}	1.7 ± 2.9

* Aphids in the treatment Febvay D were transferred to the diet after feeding on the plant for two days.

[†] Means in a column followed by different letters are significantly different at the α=0.05 level (Newman-Keuls multiple comparison procedure).

TABLE 3.

MEAN PERCENTAGE ± S.D.[†] OF SURVIVING APHIDS REARED WITH VARIOUS SACHET CHANGES THAT HAD MOLTED TO THE ADULT STAGE ON DAY 19 AND MEAN NYMPHS PRODUCED PER ADULT ± S.D.[†] DURING THE STUDY.

Treatment	requency of Sachet Change	% Adult on Day 19	Nymphs per Adult
Febvay A	Weekly	85.7 ± 14.3	0.2 ± 0.2
Febvay B	Every 2 Days	90.9 ± 11.1	0.1 ± 0.2
Febvay C	Every Day	81.4 ± 15.4	0.2 ± 0.2
Febvay D[*]	Every 2 Days	90.0 ± 4.8	0.2 ± 0.1

[*] Aphids in the treatment Febvay D were transferred to the diet after feeding on the plant for two days.

[†] Means in a column followed by different letters are significantly different at the α=0.05 level (Newman-Keuls multiple comparison procedure).

Production of nymphs began by day nine and continued until day 32. Numbers of nymphs produced per adult were calculated for each day and totaled at the end of the study. Each adult aphid on all treatments produced an average of less than 0.25 nymphs. There were no significant differences in nymphal production among the treatments.

Comparison of Aphid Survival, Development, and Fecundity on Various Food Sources

Survival, development, and nymph production were compared for aphids feeding on leaves of whole plants, explants, and the three artificial diets. Survival of aphids on the diets of Febvay and Abisgold declined over the first three days of the experiment. Aphids on these diets showed the lowest survival rates of all treatments after one week (Table 4). After six days, aphids reared on whole plants (90.0 %), explants (95.0 %), and sachets of the Akey and Beck diet (98.8 %) had significantly greater ($p < 0.05$) mean percent survivals than those on the Febvay diet (70.0 %). Percent survival of aphids reared on the Abisgold diet (78.8 %) did not differ significantly from the Febvay diet nor from the other three treatments. After 13 days, the aphids on the diet of Akey and Beck had the highest survival at 98.8%. This rate of survival was significantly higher than that for both explants, 68.8%, and Febvay diet, 68.3%. After 26 days, survival rates for aphids on all other treatments were significantly lower than that for aphids on plant leaves (57.5%). Average survival on the three diets was 20% or less at this time.

Developmental rates and nymphal production were greatly reduced by the diets in comparison to plant tissues (Table 5). On the eighth day of the study, 88 to 96% of the aphids on whole plants and explants had reached the adult stage. This rate of development was significantly faster than that for aphids reared on diets. Of the

TABLE 4.

MEAN PERCENT SURVIVAL OF APHIDS ± S.D.[†] REARED ON WHOLE PLANTS, EXCISED LEAVES, AND THREE ARTIFICIAL DIETS.

Treatment	Day 6	Day 13	Day 20	Day 26
Plant	90.0 ± 5.8^{A}	83.8 ± 11.1^{AB}	72.5 ± 9.6^{A}	57.5 ± 20.6^{A}
Explant	95.0 ± 4.1^{A}	68.8 ± 16.5^{B}	40.0 ± 9.1^{B}	28.8 ± 6.3^{B}
Febvay	70.0 ± 18.0^{B}	68.3 ± 17.6^{B}	43.3 ± 7.6^{B}	16.7 ± 5.8^{B}
Abisgold	78.8 ± 12.5^{AB}	77.5 ± 13.2^{AB}	75.0 ± 9.1^{A}	20.0 ± 10.0^{B}
Akey and Beck	98.8 ± 2.5^{A}	98.8 ± 2.5^{A}	78.0 ± 16.2^{A}	17.4 ± 15.1^{B}

[†] Means in a column followed by different letters are statistically significant at the α=0.05 level (Newman-Keuls multiple comparison procedure).

TABLE 5.

MEAN PERCENT ± S.D.† OF SURVIVING APHIDS THAT REACHED ADULT STAGE BY DAY 8 AND MEAN NYMPHS ± S.D.† PRODUCED PER ADULT APHID REARED ON CAGED LEAVES, EXCISED LEAVES, AND THREE DIET TREATMENTS.

Treatment	% Adult by Day 8	Nymphs/Adult by Day 26
Plant	88.0 ± 13.5^{A}	73.7 ± 7.2^{A}
Explant	95.9 ± 5.3^{A}	44.2 ± 7.1^{B}
Febvay	39.5 ± 27.5^{B}	$12.3 \pm 1.9^{Ca††}$
Abisgold	0.0 ± 0.0^{C}	5.8 ± 0.6^{Cc}
Akey and Beck	35.3 ± 19.0^{B}	8.5 ± 1.8^{Cb}

† Means in a column followed by different etters are significantly different at the α=0.05 level (Newman-Keuls multiple comparison procedure).

†† Means in a column followed by different lowercase letters are statistically different from each other at the α=0.05 level (Newman-Keuls multiple comparison procedure).

three artificial diets, Febvay (39.5%) and Akey and Beck (35.3%) supported a much faster rate of development than the Abisgold diet, on which no adults were found by day 8. Aphids reared on whole plants produced a mean of 73.7 nymphs per adult by day 26. Aphids on explants produced a mean of 44.2 nymphs per adult, while aphids reared on the three diets produced means of less than 15 nymphs per adult. Newman-Keuls test of reproductive rates on the three diet treatments alone revealed that the aphids on the Febvay diet (12.3 nymphs per adult) produced significantly ($P< 0.05$) more nymphs than adults reared on either Akey and Beck (8.5 nymphs per adult) or Abisgold (5.8 nymphs per adult). Aphids on the Akey and Beck diet produced significantly more nymphs per adult than those reared on Abisgold. The Akey and Beck diet and the Febvay diet were the best of the three media for aphid survival, development, and nymph production.

The Febvay diet was chosen as the base diet for future experiments, because it can be prepared in a concentrated form allowing for dilutions of serum to be added without diluting the diet nutrients. The Akey and Beck diet is a nearly saturated solution, and it could not be prepared in a concentrated form.

Feeding Trial Using Different Dilutions of Pooled Pre-Immune Rabbit Sera

To assess the degree to which non-immunized rabbit serum might have an effect on aphid survival, development and nymphal production, sera from three rabbits, C, J1, and J2, were pooled and added to the base diet (Febvay) at dilutions of 1:3, 1:6, 1:15, and 1:30. Aphids were reared on these serum dilutions and on diet only for 26 days. Table 6 reports the effect of normal rabbit sera on aphid survival over four weeks. During the first week of feeding, the content of rabbit serum in the diet caused a dosage dependent

TABLE 6.

MEAN PERCENT SURVIVAL ± S.D. OF PEA APHIDS REARED ON THE FEBVAY DIET WITH POOLED NON-IMMUNE SERUM DILUTIONS.

Serum Dilution	Day 6	Day 13	Day 20	Day26
1:3 Serum	2.5 ± 5.0	2.5 ± 5.0	2.5 ± 5.0	2.5 ± 5.0
1:6 Serum	23.8 ± 11.8	21.3 ± 12.5	20.0 ± 13.5	12.5 ± 2.9
1:15 Serum	43.8 ± 6.3	30.0 ± 14.7	23.8 ± 11.1	8.8 ± 4.8
1:30 Serum	68.8 ± 9.5	58.8 ± 4.8	35.0 ± 7.1	11.3 ± 7.5
Diet Only	70.0 ± 18.0	68.3 ± 17.6	43.3 ± 7.6	16.7 ± 5.8

mortality. Aphids reared on diet only and the 1:30 dilution of serum had the highest survival rates after six days (70.0 % and 68.8% respectively). Those on the 1:15 dilution and the 1:6 dilution had only 43.8% and 23.8% survival respectively. Aphids reared on the 1:3 dilution of serum had the lowest survival rate (2.5%) after six days. There were modest declines in survival for the next two weeks. Although not statistically significant, variations in pooled serum dilution in the diet indicated a possible inhibitory effect on development, but had no apparent influence on production of nymphs (Table 7). Approximately eight percent of aphids reared on serum dilutions of 1:6 and 1:15 had molted to the adult stage by day eight, whereas 22% and 39.5% of aphids reared on a serum dilution of 1:30 and diet only, respectively, had become adults. All serum dilutions, except for 1:3, supported the production of 12 to 15 nymphs per adult by the 26th day of the study.

Feeding Trial Using Individual Pre-Immunization Rabbit Sera

Effects of individual rabbit sera were examined by incorporation of sera from the rabbits C, J1, and J2 at dilutions of 1:15 and 1:30 into the Febvay diet. Aphids reared on serum from rabbit C suffered a higher mortality than aphids reared on serum from J1 and J2 (Table 8). On day six, aphids feeding on serum from rabbit C had mean survivals of 7.5% at 1:15 dilution and 2.6% at 1:30 dilution, while survival on the sera of J1 and J2 at 1:15 dilution were 91.1% and 91.3% and at 1:30 dilution 80.0% and 84.9% respectively. Pre-immunization sera of J1 and J2 did not differ from each other at either dilution in their effects on aphid survival. Survival of aphids reared on the C rabbit serum was insufficient for calculation of either adult development or nymphal production. There

TABLE 7.

MEAN PERCENTAGE ± S.D. OF SURVIVING APHIDS THAT REACHED THE ADULT STAGE BY DAY 8 AND MEAN NYMPHS ± S.D. PRODUCED PER ADULT APHID BY DAY 26 REARED ON THE FEBVAY DIET WITH POOLED NON-IMMUNE SERUM DILUTIONS.

Serum Dilution	% Adult by Day 8	Nymphs/Adult Day 26
Diet Only	39.5 ± 27.5	12.3 ± 1.9
Serum 1:30	22.1 ± 15.0	13.1 ± 1.5
Serum 1:15	8.1 ± 10.6	12.3 ± 1.9
Serum 1:6	8.3 ± 16.7	14.5 ± 2.1
Serum 1:3	----------	----------

TABLE 8.

MEAN PERCENT SURVIVAL ± S.D. OF APHIDS REARED ON DIET ALONE OR WITH DILUTIONS OF PRE-IMMUNE SERA OF THREE RABBITS.

Treatment	Immunization Status	Serum Conc.	Day 6	Day 12
Diet Only	N/A	N/A	92.5 ± 6.5	90.0 ± 4.1
Rabbit C	Pre	1:15	7.5 ± 6.5	3.8 ± 4.8
		1:30	2.6 ± 5.3	1.3 ± 2.6
Rabbit J1	Pre	1:15	91.1 ± 7.7	91.1 ± 7.7
		1:30	80.0 ± 19.6	78.8 ± 21.8
Rabbit J2	Pre	1:15	91.3 ± 8.5	86.4 ± 10.1
		1:30	84.9 ± 9.0	83.7 ± 8.4

were no differences between developmental rates or nymph production for aphids reared on J1 or J2 sera, but dose dependent effects of the sera were evident. Sixty-seven percent of aphids reared on J1 serum 1:30 dilution had become adults by day nine, compared to only 57% of those on the 1:15 dilution. Eighty five percent of aphids reared on diet containing the serum of rabbit J2 at 1:30 dilution had molted to the adult stage by day nine, while only 66% had developed to adults on a 1:15 dilution. Aphids reared on the 1:30 sera dilutions produced greater than one nymph per adult over the twelve days of the study, while aphids reared on the 1:15 dilution produced 0.5 nymphs per adult or less (Table 9).

Protein Assay and SDS-PAGE of Aphid Midgut Sonicate

Approximately 9,000 to 10,000 pea aphid midguts were collected. Pea aphid midguts contained an estimated 1.0 to 4.3 μg protein per gut. Midgut homogenate fractionated by SDS-PAGE revealed the presence of many proteins/ polypeptides (Figure 1). There were no major differences noted between Coomassie or silver-stained gels. Predominant stained protein/ polypeptide bands were found between 166 and 205 KDa, at 55 KDa, and in a densely stained area between 40 and 29 KDa. Numerous other bands were stained below 29 KDa. There were sufficient numbers of proteins above 10 KDa to predict that aphid gut homogenate would elicit an immune response in rabbits upon immunization with the homogenate.

Immunoblotting of Pre- and Post-immunization Sera from Rabbits C, J1, J2

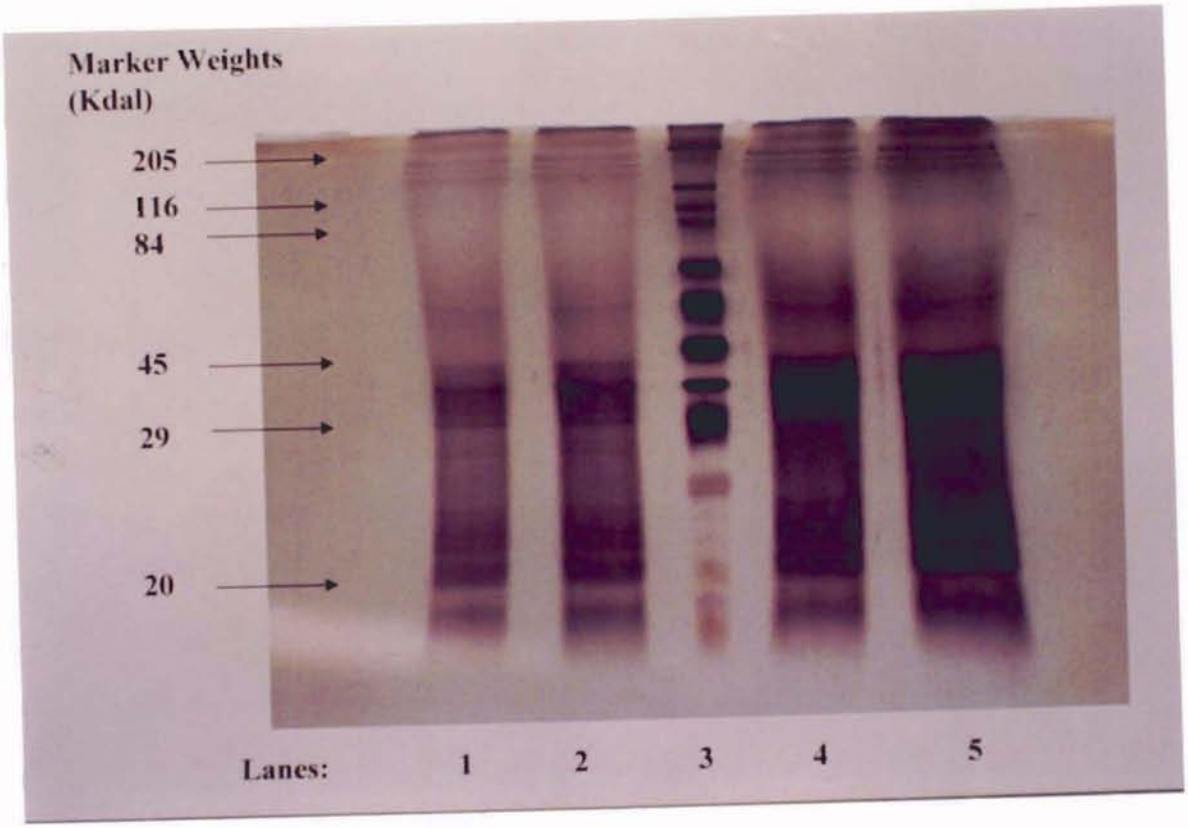
Post-immunization sera from J1 and J2 collected two weeks after completion of the initial immunization and three booster injections were immunoblotted to determine if

TABLE 9.

MEAN PERCENTAGE OF SURVIVING APHIDS THAT REACHED THE ADULT STAGE ± S.D. BY DAY 9 AND MEAN NYMPHS PRODUCED PER ADULT ± S.D. BY DAY 12 FOR APHIDS REARED ON DIET ALONE OR WITH DILUTIONS OF PRE-IMMUNE SERUM.

Treatment	Immunization Status	Serum Conc.	% Adult on Day 9	Nymphs/Adult by Day 12
Diet Only	N/A	N/A	97.2 ± 5.6	2.2 ± 1.1
Rabbit C	Pre	1:15	----------	----------
		1:30	----------	----------
Rabbit J1	Pre	1:15	56.6 ± 18.0	0.5 ± 0.3
		1:30	67.3 ± 6.5	1.1 ± 0.3
Rabbit J2	Pre	1:15	66.3 ± 12.3	0.3 ± 0.3
		1:30	85.4 ± 6.7	1.3 ± 0.4

Figure 1. Silver stained 12% acrylamide gel showing protein bands. Loaded samples of aphid gut sonicate are as follows: (Lane 1) 2.5µl, (Lane 2) 5.0µl, (Lane 3) wide range molecular weight marker, (Lane 4) 7.5µl, and (Lane 5) 10µl.



antibodies specific for aphid gut proteins had been produced by the immunized rabbits (J1 and J2) (Figure 2). Pre-immunization sera of rabbits C, J1, and J2 were used as negative controls. No serum reactivity to aphid midgut proteins was seen in any of the control immunoblots. Post-immunization sera from rabbits J1 and J2 were found to be highly reactive with aphid midgut proteins and similar in their reactivities to aphid midgut proteins. Antibody binding was most intense for the protein bands in the 116 to 205 KDa and 30 to 45 KDa molecular weight ranges (Figure 2.). Less intense reactivity was observed for bands between 45 and 116 KDa, while little reactivity occurred with proteins of less than 29 KDa.

Feeding Trials with Pre- and Post-Immunization Sera from Rabbits J1 and J2

Pre- or post-immunization rabbit sera at dilutions of 1:15 and 1:30 were incorporated into the Febvay diet and fed to aphids for 12 days to determine if the antibodies to midgut proteins would have any effects (Table 10). Differences between aphid survival on pre- and post-immunization sera of both rabbits were not significant ($p<0.05$ Student's T test) on days six or 12. Aphids reared on pre-immunization serum from rabbits J1 and J2 at dilutions of 1:15 had higher mean survivals of 91.1 % and 91.3 % than aphids reared on post-immunization sera at the same dilution, 73.8 % and 88.8 %, respectively, at day six. The opposite was true for the 1:30 dilution. Aphids fed J1 and J2 pre-immunization sera at 1:30 dilution had mean survivals of 80.0 % and 84.9 %, while aphids fed post-immunization sera dilution of 1:30 of J1 and J2 had mean survivals of 92.5% and 88.8 %. At day 12, the survival rates were similar. The experiment was terminated after 12 days because of low reserves of pre-immunization sera from rabbits

Figure 2. Western Blot of C, J1, and J2 Sera. (Far left and right strips): Wide range molecular weight markers, (2nd and 3rd): no serum, (4th and 5th): serum of non-immunized rabbit C, 1:10 and 1:25, (6th and 7th): J1 rabbit serum, 1:10 and 1:25, (8th and 9th): J2 rabbit serum, 1:10 and 1:25.

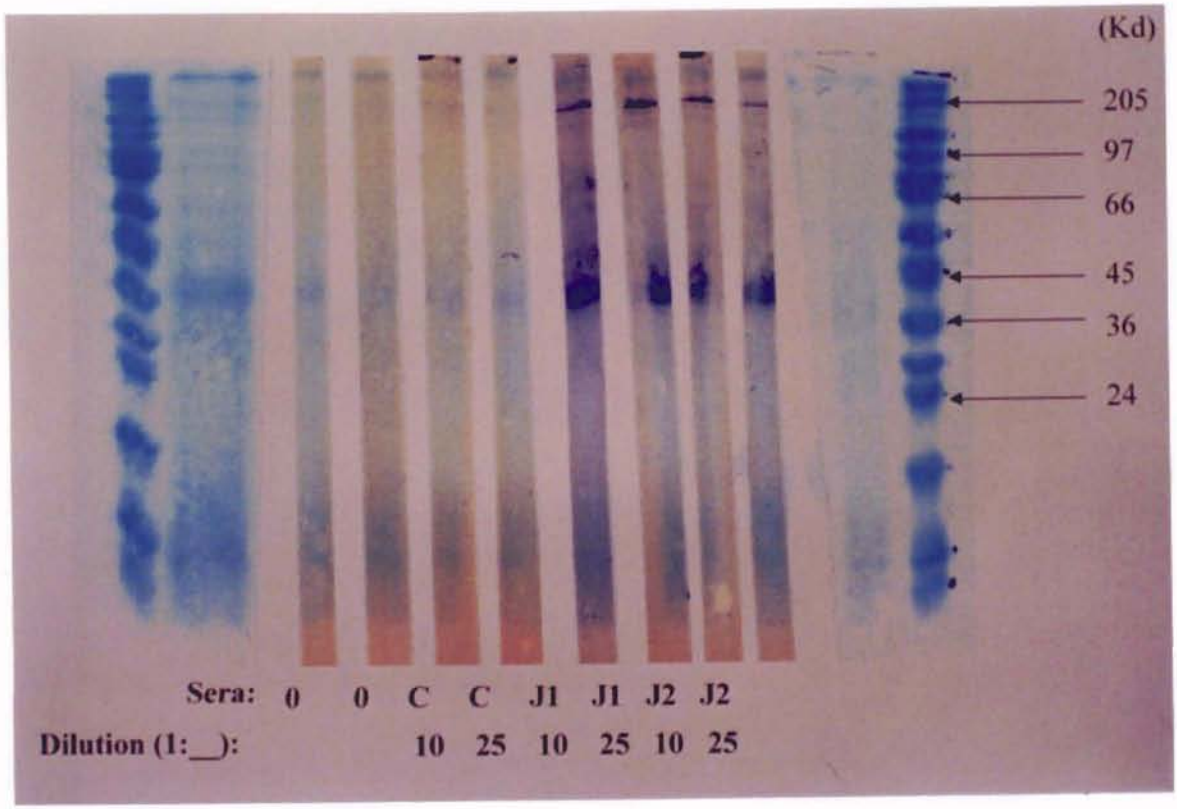


TABLE 10.

MEAN PERCENT SURVIVAL ± S.D. OF APHIDS REARED ON DIET ALONE OR WITH DILUTIONS OF PRE-IMMUNE AND IMMUNE SERUM.

Day	Serum Source & Dilution	Pre-Immunization	Post-Immunization
6	Rabbit J1 1:15	91.1 ± 7.7	73.8 ± 12.5
	1:30	80.0 ± 19.6	92.5 ± 9.6
	Rabbit J2 1:15	91.3 ± 8.5	88.8 ± 16.5
	1:30	84.9 ± 9.0	88.8 ± 11.1
12	Rabbit J1 1:15	91.1 ± 7.7	73.8 ± 12.5
	1:30	78.8 ± 21.8	86.3 ± 11.1
	Rabbit J2 1:15	86.4 ± 10.1	78.8 ± 22.9
	1:30	83.7 ± 8.4	87.4 ± 9.5

J1 and J2. The mean developmental rates of aphids reared on pre-immunization serum of rabbit J1 (1:30, 67.3 % and 1:15, 56.6 % of surviving aphids were adults) were significantly higher ($P < 0.05$) than on post-immunization sera (1:30, 90.7 % and 1:15,65.0 % of surviving aphids were adults) at either 1:15 or 1:30 dilutions (Table 11). However, post-immunization serum of the J2 rabbit caused a statistically significant ($P < 0.05$) reduction in aphid development at both the 1:15 (pre-, 66.3 % and post-, 29.6 %) and 1:30 (pre-, 85.4 % and post-, 54.2 %) serum dilutions. Similar contrasts were observed for nymph production where aphids reared on post-immunization serum of J1 (1.2 nymphs per adult), diluted 1:15, produced significantly more nymphs than did the pre-immunization serum (0.5 nymphs per adult) (Table 11). Post-immunization serum from rabbit J2 (0.2 nymphs per adult) caused a significant decrease in nymphal production at a 1:30 dilution compared to the pre-immunization serum (1.3 nymphs per adult). Subsequent experimentation revealed no significant differences between pre- and post-immunization sera of J1 and J2 at dilutions of 1:5, 1:10, and 1:15 (Table 12).

Purified IgG: Identity and Purity

IgG preparations from the sera of rabbits C, J1, and J2 were tested for the presence of IgG antibodies by double immunodiffusion with anti-rabbit IgG. Results are shown for each rabbit in Figure 3. Precipitate formed for each rabbit's IgG preparation, but titers differed. Precipitate was formed at a serum dilution of 1:3,125 for C rabbit IgG. J1 IgG had a titer of 1:125, and J2 IgG had a titer of 1:625. Differences in titer were due to the concentration of protein attained in preparation of the purified IgG

TABLE 11.

MEAN PERCENTAGE OF SURVIVING APHIDS THAT REACHED THE ADULT STAGE ± S.D. BY DAY 9 AND MEAN NYMPHS PRODUCED PER ADULT ± S.D. BY DAY 12 FOR APHIDS REARED ON DIET ALONE OR WITH DILUTIONS OF PRE-IMMUNE SERUM.

	% Adult		**Nymphs/Adult**	
Serum Source	Pre-Immunization	Post-Immunization	Pre-Immunization	Post-Immunization
Rabbit J1 1:15	56.6 ± 18.0	65.0 ± 7.7	0.5 ± 0.3	1.2 ± 0.3 *
1:30	67.3 ± 6.5	90.7 ± 7.2 *	1.1 ± 0.3	1.9 ± 1.1
Rabbit J2 1:15	66.3 ± 12.3	29.6 ± 10.2 *	0.3 ± 0.3	0.0 ± 0.0
1:30	85.4 ± 6.7	54.2 ± 24.8 *	1.3 ± 0.4	0.2 ± 0.3 *

* Means within pairs (in rows) are significantly different ($p<0.05$, Student's T-test).

TABLE 12.

MEAN PERCENT SURVIVAL ± S.D. ON DAY 6 OF APHIDS REARED ON THE FEBVAY DIET OR DIET WITH PRE OR POST-IMMUNIZATION SERA FROM TWO RABBITS.

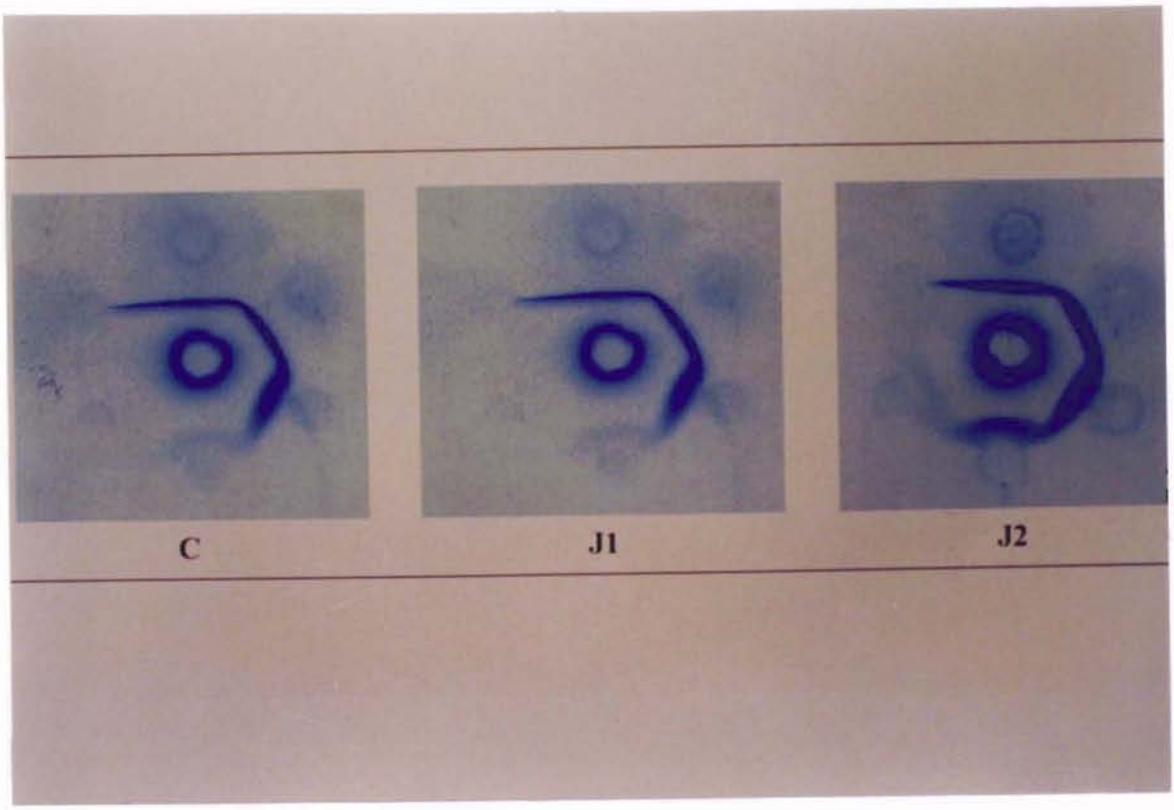
Treatment	Serum Dilution	Pre-immunization	Post-immunization
Diet Only	N/A	78.4 ± 5.2	----------
Rabbit J1	1:5	17.5 ± 13.2	30.0 ± 10.8
	1:10	32.5 ± 17.6	36.3 ± 20.2
	1:15	58.8 ± 30.1	74.6 ± 11.1
Rabbit J2	1:5	21.3 ± 14.9	23.8 ± 6.3
	1:10	62.5 ± 30.1	52.5 ± 16.6
	1:15	67.5 ± 20.0	70.0 ± 33.2

TABLE 12.

MEAN PERCENT SURVIVAL ± S.D. ON DAY 6 OF APHIDS REARED ON THE FEBVAY DIET OR DIET WITH PRE OR POST-IMMUNIZATION SERA FROM TWO RABBITS.

Treatment	Serum Dilution	Pre-immunization	Post-immunization
Diet Only	N/A	78.4 ± 5.2	----------
Rabbit J1	1:5	17.5 ± 13.2	30.0 ± 10.8
	1:10	32.5 ± 17.6	36.3 ± 20.2
	1:15	58.8 ± 30.1	74.6 ± 11.1
Rabbit J2	1:5	21.3 ± 14.9	23.8 ± 6.3
	1:10	62.5 ± 30.1	52.5 ± 16.6
	1:15	67.5 ± 20.0	70.0 ± 33.2

Figure 3. Coomassie stained double immunodiffusion gels. Outer wells were filled with a dilution of the IgG preparation of each rabbit (C, J1, and J2). Dilutions: top well 1:1, clockwise 1:5, 1:25, 1:125, 1:625, and 1:3125. The center well contained anti-rabbit IgG.



IgG preparations were next tested for purity by immunoelectrophoresis (IEP) (Figure 4). Electrophoresis of the IgG preparations followed by diffusion versus anti-whole rabbit serum revealed two precipitin arcs each for IgG preparations of J1 and J2. Three arcs were formed in IEP of the C rabbit IgG preparation. A precipitin arc corresponding to the IgG component was identified for each rabbit's IgG preparation.

Effects of Purified IgG or Whole Serum

Preparations of purified IgG from the sera of rabbits C (unimmunized), J1 and J2 (immunized) were compared to whole sera of these rabbits to determine whether or not killing effects of sera were due to the IgG component. Purified IgG protein contents were determined by BCA protein microassay to be 15 mg per ml for rabbit C, 5.0 mg per ml for rabbit J1, and 4.5 mg per ml for rabbit J2. IgG preparations were added to the Febvay diet at the appropriate protein concentration corresponding to the IgG content of whole serum at dilutions 1:9, 1:15, and 1:30. Aphids were then reared on a diet containing whole serum or the corresponding protein concentrations of purified IgG. All IgG preparations were found to be extremely toxic to aphids at all concentrations tested over a three week period (Table 13). Initially, over the first four to six days, aphids on the IgG preparation of J1 and J2 did not experience a drop in survival, and survivals at a dilution of 1:9 were very near that of diet alone (Figure 5). Similar effects were seen in aphids fed the C rabbit IgG, but the drop in survival came after only two days. Aphids reared on whole sera did however experience a drop in survival over the first three days typical of that previously noted for aphids fed whole serum.

Figure 4. Buffalo black stained immunoelectrophoretic gels. Purified IgG preparations from sera of rabbits C, J1, J2 were used as the samples. Polyvalent anti-whole rabbit serum was used as the anti-sera challenge.

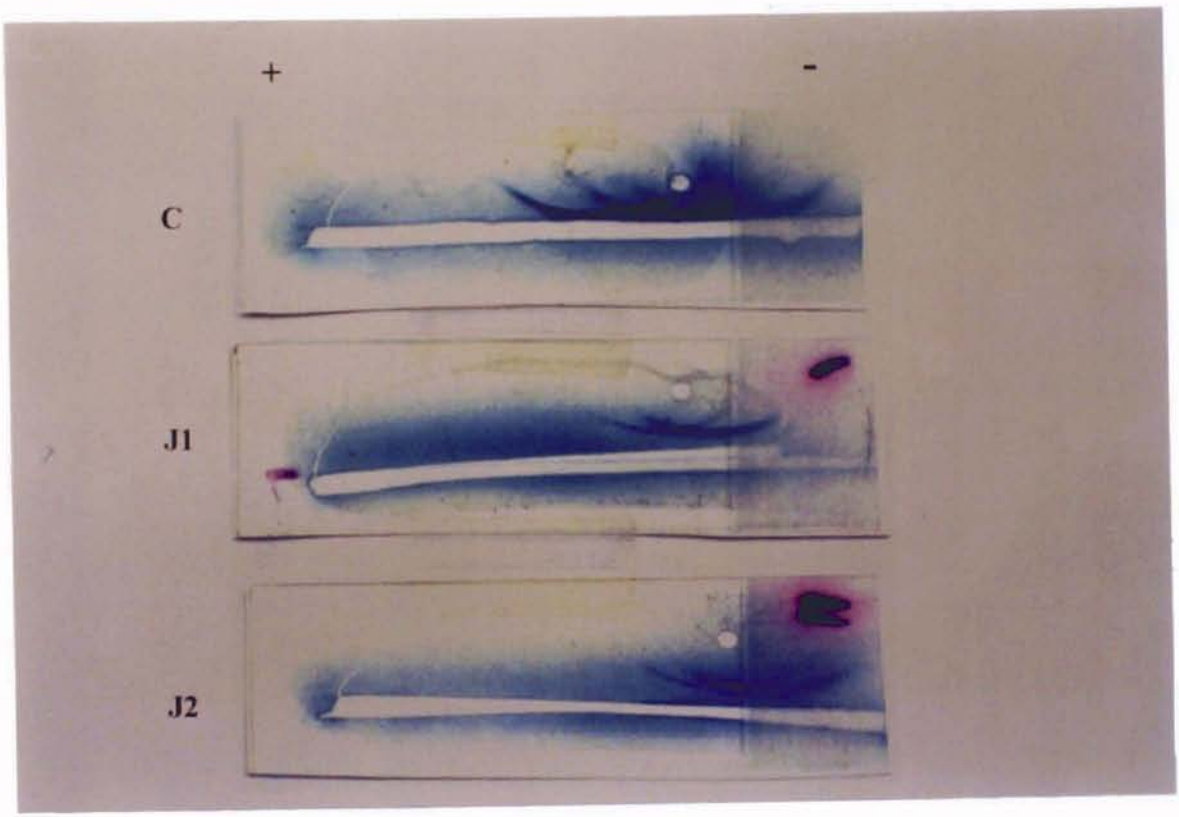


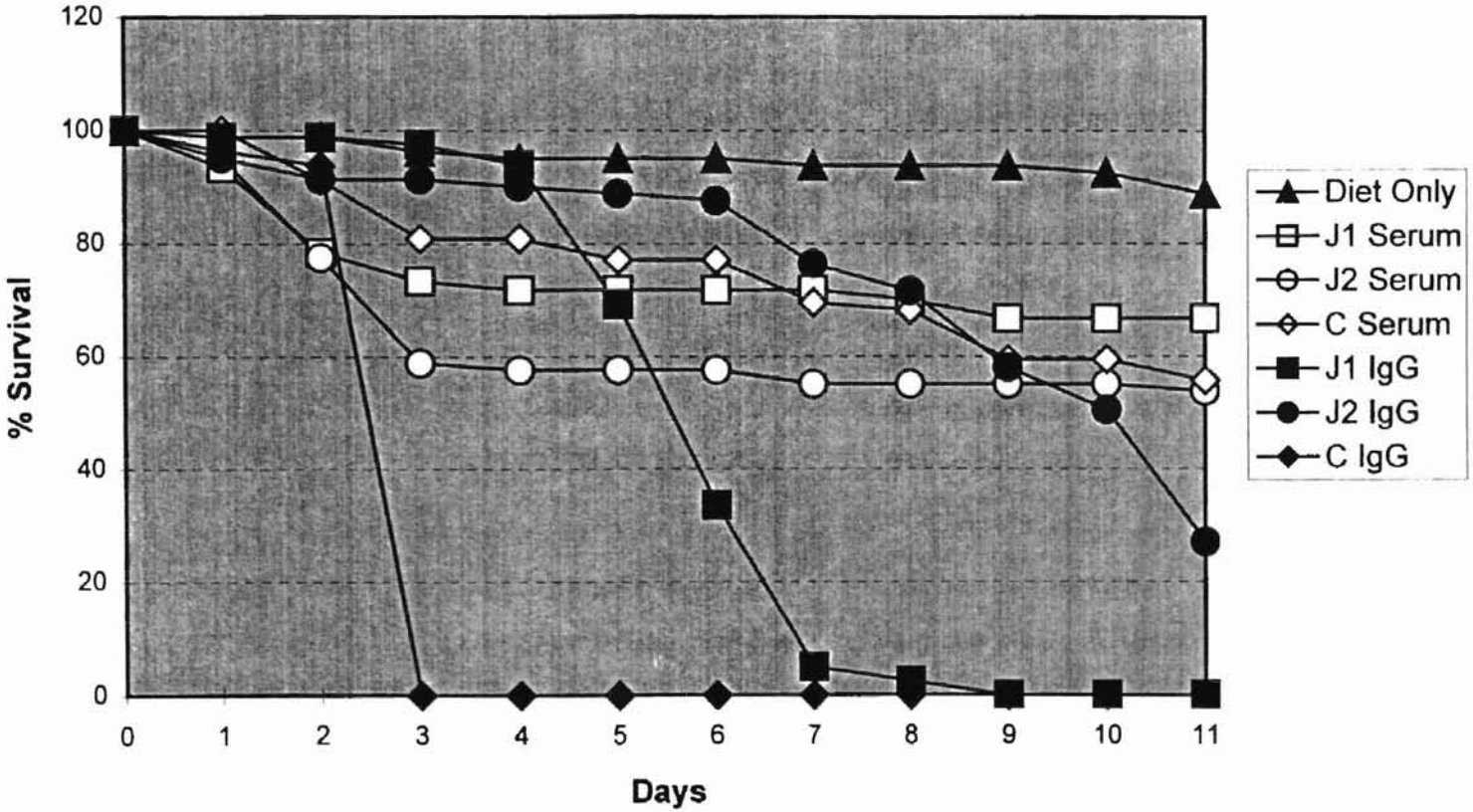
TABLE 13.

MEAN PERCENT SURVIVAL ± S.D. OF APHIDS REARED ON THE FEBVAY DIET WITH WHOLE SERUM (1:9, 1:15, AND 1:30 DILUTIONS) OR THE EQUIVALENT PROTEIN CONCENTRATION OF IgG BATCH PURIFICATION PREPARATION.

		1:9 Serum Dilution		**1:15 Serum Dilution**		**1:30 Serum Dilution**	
Day	Serum Source	Serum	IgG Preparation	Serum	IgG Preparation	Serum	IgG Preparation
6	Nonimmunized C	77.0 ± 11.2	0.0 ± 0.0 *	------	0.0 ± 0.0	95.1 ± 4.1	0.0 ± 0.0 *
	Immunized J1	71.7 ± 5.8	35.5 ± 11.5 *	66.3 ± 16.0	95.1 ± 3.9 *	86.3 ± 7.5	95.0 ± 4.1
	Immunized J2	57.5 ± 23.6	87.6 ± 6.6	81.3 ± 7.5	81.7 ± 15.3	85.2 ± 6.9	91.7 ± 7.6
13	Nonimmunized C	33.1 ± 11.4	0.0 ± 0.0 *	------	0.0 ± 0.0	50.7 ± 6.6	0.0 ± 0.0 *
	Immunized J1	51.7 ± 7.6	0.0 ± 0.0 *	40.0 ± 10.0	1.3 ± 2.5 *	50.0 ± 12.3	3.8 ± 2.5 *
	Immunized J2	50.0 ± 23.5	8.6 ± 7.3 *	52.5 ± 6.5	3.3 ± 5.8 *	39.6 ± 7.8	3.3 ± 5.8 *
20	Nonimmunized C	21.7 ± 13.8	0.0 ± 0.0	------	0.0 ± 0.0	21.0 ± 4.5	0.0 ± 0.0 *
	Immunized J1	30.0 ± 13.2	0.0 ± 0.0	20.0 ± 7.1	0.0 ± 0.0 *	16.3 ± 4.8	0.0 ± 0.0 *
	Immunized J2	32.5 ± 9.6	0.0 ± 0.0 *	33.8 ± 4.8	0.0 ± 0.0 *	11.3 ± 8.5	0.0 ± 0.0

* Means within pairs of the same dilution (in rows) are significantly different ($p<0.05$, Student's T-test).

Figure 5. Survival of Pea Aphids on Diet Containing a 1:9 Dilution of Serum or the Equivalent IgG Content



Screening of Rabbit Sera for Low Aphid Toxicity

The differences between sera effects of individual rabbits and the need for a larger supply of pre-immunization serum prompted the screening of sera from other rabbits to find sera with low innate toxicity to aphids. Aphids were reared on diet containing pre-immunization sera from five rabbits at dilutions of 1:5, 1:15, and 1:30 for six days. Analysis of variance with a Newman-Keuls test for each serum dilution showed no significant differences between survival rates for aphids feeding on the different rabbit sera at the 1:5 and 1:15 serum dilutions. However, at the 1:30 dilution, aphid survival on the B1 rabbit serum (45.0 %) was significantly less than for the A3 rabbit serum (93.3 %)(Table 14). Rabbits A2, A3, and B2 were chosen for immunization based on greater survival of aphids fed on 1:30 dilutions of their pre-immunization sera.

Immunoblots of Pre- and Post-Immunization Sera of Rabbits A2, A3, and B2

Sera collected from rabbits A2, A3, and B2 after the initial immunization and four booster injections were evaluated for antibody reactivity to aphid midgut proteins by immunoblot analysis. Pre-immunization sera from the rabbits showed no reactivity. Post-immunization sera contained antibodies reactive to aphid midgut polypeptides of several molecular weights (Figure 6). Results were similar to the earlier immunoblots of J1 and J2. Antibody binding again occurred in the ranges of 166 to 205 KDa and 30 to 45 KDa, with less reactivy between 45 and 166 KDa.

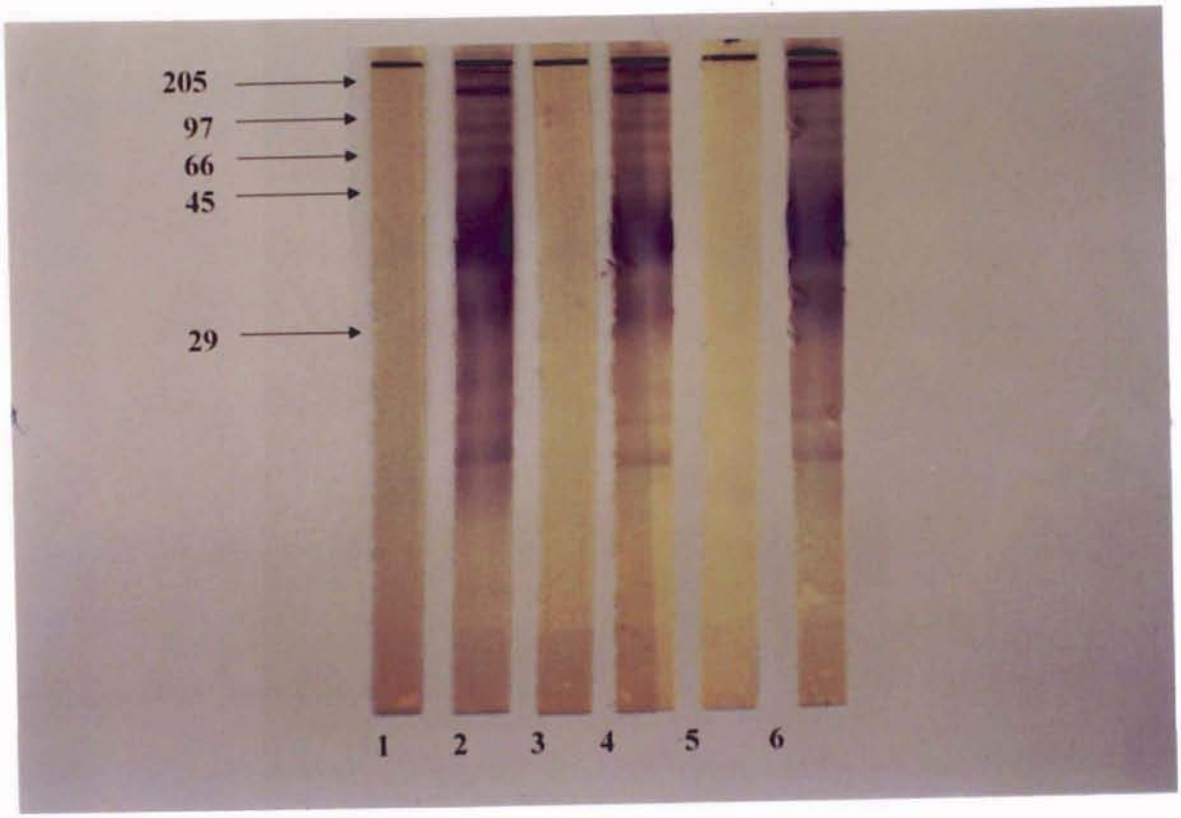
TABLE 14.

MEAN PERCENT SURVIVAL ± S.D.[†] ON DAY 6 OF APHIDS REARED ON THE FEBVAY DIET OR DIET WITH PRE-IMMUNIZATION SERA FROM ONE OF FIVE RABBITS.

Dilution	Diet Only	A2	A3	B1	B2	B3
No Serum	85.0±5.0					
1:5		11.7 ± .5.8	30.0 ± 2.9	10.0 ± 5.0	6.7 ± 2.9	10.0 ± 13.2
1:15		58.3 ± 7.6	68.3 ± 20.8	78.3 ± 11.6	48.3 ± 20.8	68.3 ± 14.4
1:30		71.7 ± 2.9^{AB}	93.3 ± 2.9^{A}	45.0 ± 5.0^{B}	70.0 ± 8.0^{AB}	63.3 ± 5.2^{AB}

[†] Means in a row followed by different letters are significantly different at the α=0.05 level (Newman-Keuls multiple comparison procedure).

Figure 6. Western Blot of pre- and post-immunization sera of rabbits A2, A3, and B2. (Strips 1 and 2): Pre- and post-immunization sera of rabbit A2, (Strips 3 and 4): Pre- and post-immunization sera of rabbit A3, and (Strips 5 and 6): Pre- and post-immunization sera of rabbit B2. First antibody diluted 1:50.



Feeding Trial with Pre- and Post-Immunization Sera of Rabbits A2, A3, and B2

Comparisons of pre- and post-immunization sera effects on survival revealed a significant decrease ($P < 0.05$) in survival on the post-immunization serum treatments of A2, and A3 at a 1:9 dilution (Table 15). Aphids reared on the pre-immunization sera of A2 and A3 at a 1:9 dilution had survival ratess of 81.4 % and 83.8 %, respectively, at day six, and aphids fed post-immunization sera had survivals of 37.5 % and 40.0 %. Both A2 and A3 post-immunization rabbit sera were similar in their effects on aphid survival (Figure 7). Aphids reared on the 1:9 dilutions of pre- (48.3 %) and post-immunization serum (56.3 %) of the B2 rabbit showed no such effect. Effects of pre- and post-immunization sera from all rabbits were lessened or lost at dilutions of 1:15 and 1:30. Generally development and nymphal production were not statistically different between pre- and post-immunization sera. However, aphids reared on a 1:9 dilution of post-immunization serum of rabbit A2 produced significantly more nymphs than did aphids reared on pre-immune serum (Table 16).

Differences in antibody specificities and magnitude of these specificities between rabbits were determined by densitometry tracings of immunoblots. Densitometry tracings indicated no obvious differences among rabbits in regard to antibody specificity for aphid midgut proteins or magnitude of binding.

The enzyme linked immunosorbent assay technique (ELISA) was employed to detect any differences between rabbits in their overall magnitude of the antibody response. Antibody levels of post-immunization sera from rabbits A2, A3, and B2 were much higher than the pre-immunization sera of these rabbits with mean optical density levels of 1.008 for the post-immunization sera and zero for the pre-immunization sera at

TABLE 15.

MEAN PERCENT SURVIVAL ± S.D. OF APHIDS REARED ON THE FEBVAY DIET WITH WHOLE PRE- OR POST-IMMUNIZATION SERA (1:9, 1:15, AND 1:30 DILUTIONS).

		1:9 Serum Dilution		**1:15 Serum Dilution**		**1:30 Serum Dilution**	
Day	Rabbit	Pre-immunization	Post-immunization	Pre-immunization	Post-immunization	Pre-immunization	Post-immunization
6	A2	81.4 ± 11.2	37.5 ± 8.7*	92.5 ± 6.5	81.3 ± 14.4	96.1 ± 5.0	95.0 ± 4.1
	A3	83.8 ± 13.2	40.0 ± 12.9*	90.0 ± 10.8	80.0 ± 14.7	83.8 ± 9.5	91.3 ± 7.5
	B2	48.3 ± 16.5	56.3 ± 28.7	81.3 ± 10.3	76.3 ± 4.8	96.3 ± 7.5	75.2 ± 14.3
13	A2	66.6 ± 8.8	31.3 ± 13.2*	52.5 ± 15.0	55.0 ± 20.4	60.8 ± 10.9	63.8 ± 4.8
	A3	66.3 ± 15.5	35.0 ± 7.1*	49.5 ± 10.2	63.8 ± 11.1	58.8 ± 7.5	51.3 ± 9.5
	B2	42.0 ± 19.1	50.0 ± 25.5	51.3 ± 11.1	72.5 ± 2.9*	42.5 ± 11.9	49.3 ± 8.1
20	A2	30.7 ± 9.4	18.8 ± 13.2	17.5 ± 2.9	23.8 ± 21.7	22.8 ± 13.3	26.3 ± 7.5
	A3	30.0 ± 8.2	18.8 ± 4.8	20.4 ± 8.8	27.5 ± 15.0	30.0 ± 8.2	26.3 ± 4.8
	B2	35.0 ± 10.0	28.8 ± 14.9	18.8 ± 9.5	32.5 ± 9.6	8.8 ± 2.5	24.7 ± 0.6*

* Means within pairs of the same dilution (in rows) are significantly different ($p<0.05$, Student's T-test).

Figure 7. Pea aphid survival on artificial diet containing pre- and post-immunization sera of rabbits A2 and A3

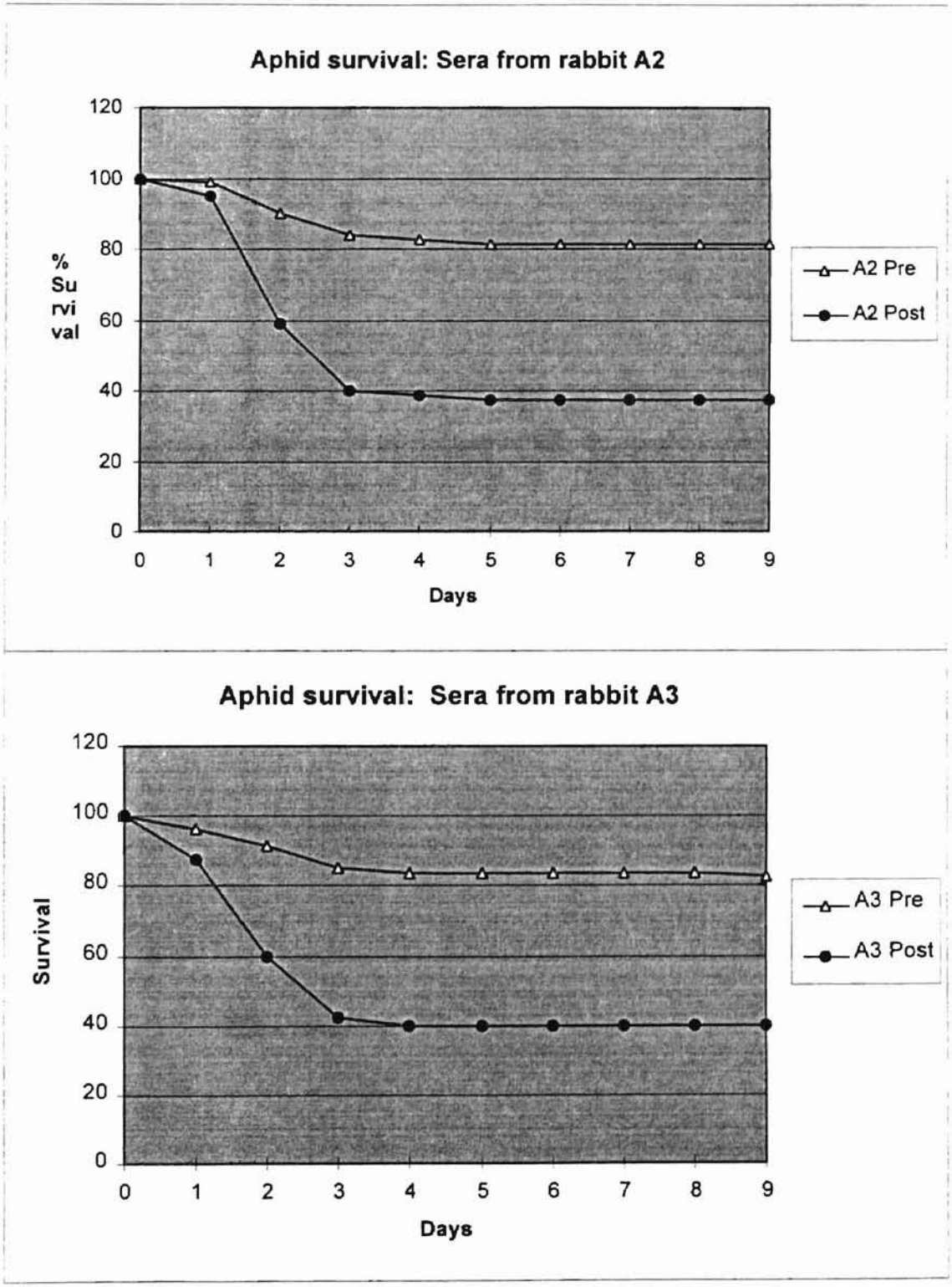


TABLE 16.

MEAN PERCENTAGE ± S.D. OF SURVIVING APHIDS THAT REACHED THE ADULT STAGE BY DAY 10 AND MEAN NYMPHS/ ADULT BY DAY 20 ON THE FEBVAY DIET WITH WHOLE PRE- OR POST-IMMUNIZATION SERA FROM THREE RABBITS (1:30, 1:15, 1:9 DILUTIONS).

		% Adult		**Nymphs/Adult**	
Serum Conc.	Rabbit	Pre-immunization	Post-immunization	Pre-immunization	Post-immunization
1:9	A2	46.4 ± 16.3	35.1 ± 15.5	6.7 ± 1.1	8.6 ± 0.7*
	A3	46.5 ± 7.7	22.5 ± 26.0	6.6 ± 0.7	7.7 ± 1.1
	B2	33.9 ± 23.8	37.4 ± 15.2	7.6 ± 2.2	7.1 ± 0.8
1:15	A2	68.4 ± 3.5	61.8 ± 9.8	6.6 ± 1.0	7.3 ± 1.4
	A3	61.6 ± 5.7	61.0 ± 21.7	7.8 ± 1.7	7.9 ± 2.3
	B2	71.6 ± 6.8	75.2 ± 8.9	6.8 ± 1.6	7.4 ± 1.2
1:30	A2	77.4 ± 4.6	67.9 ± 9.7	7.5 ± 1.4	7.0 ± 1.2
	A3	84.7 ± 7.7	81.3 ± 14.1	7.3 ± 0.6	7.2 ± 0.4
	B2	69.7 ± 32.2	88.4 ± 6.2	6.6 ± 1.8	8.2 ± 1.3

* Means within pairs (rows) are significantly different ($p<0.05$, Student's T-test).

1:40,960 dilution. Among individual rabbit sera, rabbit B2 had a higher pre-immunization antibody level (0.189) than rabbits A2 (0.005) and A3 (0.023) at 1:640 dilution. Post-immunization antibody levels of rabbits A2, A3, and B2 were 0.867, 1.135, and 1.022, respectively, at a 1:40,960 dilution.

CHAPTER IV

DISCUSSION AND CONCLUSIONS

Use of antibodies against a phytophagous insect pest species is a novel approach for pest management and may have great economic benefit. In this research, a bioassay system was successfully created to evaluate the effects of antibodies on aphids. Aphids reared in this system were able to survive, develop and reproduce. Diet sachets must be changed frequently, but no more frequently than every other day. A longer period of time between sachet changes may lead to degradation of the diet, which may become unsuitable for support of aphid development. Allowing nymphs to feed an extra day on the plant is not effective in achieving greater survival, shortening developmental time, or enhancing nymph production. This eliminates the need to remove all adult aphids from the plant while leaving the 0-24 hour old nymphs to feed an extra day.

No artificial diet tested was adequate to support aphid survival, development, or fecundity to the degree provided by plants or explants. The diets of Akey and Beck (1972) and Febvay et al. (1992) did, however, provide sufficient aphid survival, development, and fecundity for bioassays up to four weeks in duration. The Febvay et al. (1992) diet was chosen of the two as the preferable diet for this series of experiments, due to the ease in which serum could be added to the diet without altering the composition of nutrients. A consistent diet formulation is important so that differences between serum dilutions may be directly correlated to the serum in the diet and not a reduction or concentration of diet nutrients.

Counting adult aphids based on identifying them by the presence of the elongated pygidium was determined to be a more effective and easier approach for determining the developmental stage than counting exuviae (preliminary study). Counting exuviae was tedious, and conclusions about developmental time were based upon absence of cast skins rather than presence of an obvious morphological characteristic. Adult aphids were easily identified, and counting adults as they molted provided a reasonable estimate of developmental time.

Production of anti-parasitic arthropod antibodies has been successfully accomplished, using preparations of arthropod midgut (Wikel, 1988; Wikel et al., 1992). Although phytophagy and animal parasitism are very different systems, anti-aphid antibodies were successfully generated by immunization of rabbits with sonicated midgut tissues of the pea aphid. Anti-aphid activity was confirmed and characterized by immunoblot. Intense antibody binding was found for midgut proteins in the ranges of 116 to 205 KDa and 30 to 45 KDa with lesser reactivity in the 45 to 116 KDa range. Little reactivity occurred for proteins less than 29 KDa, presumably due to the small size of the proteins. Smaller molecular weight proteins, generally less than 25 KDa are less immunogenic than larger or more complex proteins (Janeway and Travers, 1994), although many smaller proteins may be quite immunogenic.

Results of feeding pooled pre-immunization sera to aphids indicated that a factor, or factors, present in rabbit sera were toxic to aphids. This may be characteristic of rabbit sera in general. Similar effects were seen in a study on mosquitoes. Mosquitoes, fed on non-immune rabbit sera, suffered a 22 % mortality rate (Srikrishnaraj et al., 1995).

Survival of pea aphids fed pooled non-immune rabbit sera were affected in a dosage dependent manner. After further investigation, a majority of the toxicity found in the pooled sera could be attributed to the serum of one rabbit (C). All rabbit sera (C, J1, and J2) were found to have some effect on aphid survival, but the magnitude of these effects varied among rabbits. This observation was confirmed by the screening of sera from five other rabbits (A2, A3, B1, B2, and B3). Due to this variability of sera toxicity among individual rabbits, rabbits should be screened for innate toxicity within an acceptable level, and their sera should be evaluated separately when used in a bioassay system for determining antibody effects. The mortality causing factor or factors present in normal rabbit sera should be investigated in more detail. Testing serum fractions, or individual serum molecules, may yield other, perhaps more potent, insecticidal molecules than antibodies.

Post-immunization sera of rabbits were shown to cause decreased survival, developmental delay, and reduction of fecundity. However, these effects were not found in sera from all rabbits, or all effects found in a single rabbit serum. Decreased survival of aphids was caused by sera from rabbits A2 and A3. Inhibition of development and reduced fecundity were caused by serum from rabbit J2. Post-immunization serum from rabbit J1 appeared to speed up development and increase nymph production and the serum of B2 appeared to allow greater survival that pre-immunization serum at a 1:9 dilution in diet. Variability among rabbit immune responses may help explain these differing effects. Significant variation has been found among immunized animals in their abilities to provide protection against arthropods (Johnston et al., 1986; Jacobs-Lorena and Lemos, 1995). Immunization of fifteen cattle with an extract of whole tick,

excluding the cuticle, yielded only seven cattle highly resistant to *B. microplus* infestation (Johnston et al., 1986). Five cattle showed intermediate resistance and the remaining three showed little or no resistance compared to controls. Anti-tick antibody levels of resistant cattle determined by gel diffusion and radio-immunoassay could not account for resistance to ticks (Johnston et al., 1986). Similar results were found in the study of anti-pea aphid antibodies produced by rabbits. Densitometry and ELISA data did not confirm any obvious differences among the rabbits A2, A3, and B2 in antibody binding or antibody response, but variation among rabbits in immune response cannot be ruled out.

Differences among rabbits in production of aphid resistant antibodies may not be as serious a problem as occurs due to immunological variation among animals in the search for an anti-parasitic arthropod vaccine. An effective antibody for control of pea aphids need not be generated in all rabbits for efficacy, as it must be if all vaccinated animals are to be protected from parasitic arthropods. Only those rabbits which produce protective antibodies against aphids are needed for further investigation.

The IgG preparations tested were found to be extremely toxic to aphids. The toxicity of IgG preparations to aphids was most likely due to a factor or factors associated with the purification process. The dosage dependent reduction in survival caused by whole serum generally occurred within the first three days of feeding. However, the toxicity of the J1 and J2 IgG preparations occurred from 4 to 6 days. On the days before the toxic effects became evident, aphid survival on purified IgG was equivalent to that on diet alone. Due to the acute toxicity of the IgG preparations in this study, another method for purification of IgG should be used, or perhaps a more extensive period of buffer exchange should be included to eliminate residual toxic effects.

These studies confirm that control of phytophagous species using antibodies is a viable approach. Other species of aphids and other sap-sucking Homoptera may be good candidates for antibody-based resistance and should be investigated in a similar manner. Further investigations regarding antibodies and other serum factors must be done before production of a transgenic plant expressing antibodies to confer resistance to pea aphids is pursued. A specific antigen or antigens which cause protective antibodies to be produced must be identified, isolated, and tested. Detergent extraction, gel permeation chromatography, and preparative isoelectric focusing have been used to successfully isolate protective antigens of *B. microplus* (Willadsen et al., 1988). Similar techniques might be used for isolation of pea aphid antigens.

Damage to plant tissues or effects on non-target species should be avoided. An almost limitless array of mammalian antibodies can be formed to bind with a vast number of molecules (Janeway and Travers, 1994), therefore, care should be taken in determining the specific mode of action of antibodies conferring insect resistance. The specific antigens to which insecticidal antibodies bind must be isolated and determined to be common only to the insect species targeted. Antibodies may bind and interfere with proteases, enzymes, chemoreceptors, or neuropeptides (Elvin and Kemp, 1994). Some antibodies may cross-react with related antigens (Janeway and Travers, 1994). Concern has already been expressed about the use of protease inhibitors in transgenic plants due to their effects on honeybees (Burgess et al., 1996). An antibody acting to inhibit a common protease would most likely affect honeybees as well.

The binding array of antibodies also has advantages. Since antibodies can bind to any number of bioactive molecules in an insect system, specific modes of action can be

sought. Proteases, enzymes, chemoreceptors, and neuropeptides may all be targets of anti-insect antibodies, as well as, specific gut associated antigens. Insect antibody targets of transgenic plants need not be confined to the easily reached midgut antigens. Ben-Yakir and Shochat (1996) reported a certain quantity of ingested IgG passed into the hemolymph of European corn borer, *Ostrinia nubilalis* (Hübner), when the larvae fed on artificial diet containing hyper-immune serum of rabbits. Similar observations were reported in parasitic arthropods (Ackerman et al., 1981; Ben-Yakir, 1989). The use of antibodies for control of insects may have broad application.

For an antibody-expressing transgenic plant approach to confer resistance against aphids, antibodies must be expressed in the plant in a manner whereby aphids would ingest the antibodies. Aphids feed in the phloem of plants, therefore antibodies must also be expressed in the phloem. Transgenic production of antibodies and other proteins by plants can be targeted to any plant organ or subcellular compartment (Hiatt, 1990). The key is to use the correct promoter sequence. The cauliflower mosaic virus (CaMV) 35S promoter can target the expression of a gene to the phloem tissue (Jefferson et al., 1987) as can the maize sucrose synthase-1 promoter (Yang and Russell, 1990) and the rice sucrose synthase-1 promoter (Shi et al., 1994; Hilder et al., 1995). The rice sucrose synthase-1 promoter has been used to direct the expression of an insecticidal lectin to the phloem, and the transgenically expressed lectin conferred resistance against aphids (Hilder et al., 1995). These and other promoters may be useful in future efforts to control pea aphids and other sap-sucking insects by expression of anti-insect antibodies.

The research reported here is only a beginning. According to literature found, this is the first report of resistance to a phytophagous species conferred by antibodies. The

levels of detrimental effects on aphids caused by antibodies presented in this study are insufficient to act as a single control mechanism in transgenic plants on a commercial scale. However, the use of these antibodies to control pea aphids in an integrated pest management setting is potentially feasible. More research in the area of insect-resistant antibody expressing transgenic plants must be performed before a commercially viable plant can be transformed and marketed.

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