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A diphasic immune response against bacteria in the American cockroach

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SUMMARY

The adult cockroach generates an adaptive humoral immune response exhibiting specificity and immunological memory when immunized with soluble proteins. In contrast, the response induced by bacteria in holometabolous insects is non-specific and short term, generally losing activity after 72 hr. We have found that the roach generates a diphasic response when injected with bacteria, displaying an acute non-specific phase initially, which is then superseded by a second response that is relatively long term and specific. Animals were immunized with either killed *Pseudomonas aeruginosa* or pyrogen-free Burns–Tracey saline (BTS) and challenged at various times post-immunization (p.i.) with a lethal dose of viable *P. aeruginosa*. The induction of immunity was measured by monitoring the percentage of survivors after challenge. Immunization with killed *P. aeruginosa* induced significant ($P < 0{\cdot}05$) protection against challenge as compared with BTS controls. The response was elicited by Day 1 p.i. and did not begin to decline until after Day 14 p.i. The specificity of the response was tested by immunizing animals with either killed *Serratia marcescens*, *Enterobacter cloacae*, *Streptococcus lactis* or *Micrococcus lysodeikticus*, and then challenging them with live *P. aeruginosa*. Significant protection against *P. aeruginosa* challenge was induced by any of the bacteria within the first 3 days p.i. However, by Day 4 the response began to show specificity. Immunization with *P. aeruginosa* induced significantly more protection than immunization with any one of the other organisms, and only the Gram-negative organisms (*P. aeruginosa, E. cloacae* and *S. marcescens*) induced any protection relative to controls. As the response continued, it became even more specific, since only immunization with *P. aeruginosa* was still protective against a lethal challenge by Day 7 p.i. Thus, the cockroach generates a diphasic antibacterial response composed of an acute, short-term, non-specific phase, as well as a more long-term, specific phase.

INTRODUCTION

Insects have evolved both cellular and humoral defence mechanisms that provide protection against infectious micro-organisms (reviewed in refs 1 and 2). Cellular responses to infections in the haemocoel initially result in phagocytosis of the foreign particles. Nodule formation and encapsulation of invaders occur later in the cellular response if phagocytosis alone cannot clear the infection.[1] The humoral response to bacteria in insects has been extensively characterized in pupae and larvae of holometabolous insects (insects that undergo complete metamorphosis). Two major families of inducible antibacterial proteins have been identified in the haemolymph of holometabolous insects. The protective properties of the so-called cecropins and attacins are short-lived, exhibiting peak activity within 72 hr of infection and then steadily decreasing.[1,2] Like the cellular responses to bacteria in insects, cecropins and attacins react non-selectively.[3,4]

Unlike the antibacterial response of holometabolous insects, the American cockroach (*Periplaneta americana*) generates an immune response to soluble proteins that displays specificity and immunological memory.[5,6] The ability to generate an adaptive immune response would be advantageous to the roach because of its longer lifespan (3 years or more). It was of interest to us to determine whether the antibacterial response in the roach possesses characteristics of the short-lived holometabolous type of response, or if it has evolved characteristics of true immunity that would contribute significantly to its longevity.

The studies presented here investigated the kinetics and specificity of the response elicited by bacteria in the American cockroach. Animals were immunized with various genera of bacteria and challenged with a lethal organism, *Pseudomonas aeruginosa*. Survival after challenge was used as a measure of induced immunity. Our results indicated that the response consisted of a non-specific, short-lived phase as well as a more sustained phase resembling true immunity.

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MATERIALS AND METHODS

Animals

Male cockroaches, less than 2 months into adulthood, were obtained from our colony stocks. Animals were housed in

Table 1. Comparative specificity of the antibacterial response: significance of immunizing with various bacteria relative to BTS-injected controls

Days post-immunization	*E. cloacae*	*S. marescens*	*M. lysodeikticus*	*S. lactis*	*P. aeruginasa*
1	0·1	0·025	0·025	0·1	0·025
2	<0·05	0·025 < *P* < 0·05	0·025	0·1	0·025
3	0·025	0·025	0·025	0·025	0·025
4	>0·1	>0·1	>0·1	>0·1	0·025
5	0·05 < *P* < 0·1	0·1	>0·1	>0·1	0·025
6	>0·1	0·025 < *P* < 0·05	>0·1	>0·1	0·025
7	>0·1	>0·1	>0·1	>0·1	0·025
14	<0·05	>0·1	>0·1	>0·1	0·025
21	—	—	—	—	0·1
28	—	—	—	—	0·1

The *P*-value for the difference in mean percentage survival after challenge is given for each group relative to the mean percentage survival resulting from immunization with BTS. Days post-immunization (p.i.) refers to the day challenged p.i.

covered 1-gallon plastic containers lined with white petrolatum to prevent escape. Roaches were fed VCA Foods canine lab diet (Cincinnati, OH) and water *ad libitum*, and were maintained at 28° on a 12/12 hr light/dark cycle.

Bacteria

P. aeruginosa (strain P11-1) was generously provided by Dr J. S. Chadwick (Queens University, Kingston Ontario, Canada). Lyophilized *Micrococcus lysodeikticus* (ATCC 4698) was purchased through Sigma (St Louis, MO). All other species of bacteria were obtained from the Biological Sciences Department, University of Cincinnati. Bacteria used for immunizations were killed in 3% glutaraldehyde in 0·1 M NaH_2PO_4, PH 7·3, for 30 min, and washed twice in sterile 0·95% NaCl. Samples of treated bacteria were plated on trypticase soy agar (TSA) to ensure that the killing technique was effective. Total bacterial cell counts were obtained by using a Petroff–Hausser counting chamber under phase-contrast optics. Cells were resuspended in sterile Burns–Tracey saline (BTS)[7] to a final concentration of 10^8 cells/ml. *P. aeruginosa* used for challenge was grown in trypticase soy broth to an optical density of approximately 85 units on Klett–Summerson colorimeter with a red filter. This density corresponded to approximately 5×10^8 CFU/ml.

Immunization and challenge procedures

Roaches were anaesthetized with CO_2, and 1×10^6 fixed bacterial cells per g of body weight or 10 μl of BTS per g of body weight (controls) was injected into the haemocoel with a Hamilton microsyringe. BTS was made with pyrogen-free water, since Dunn *et al.*[8] demonstrated that soluble peptidoglycan fragments stimulate a response in the holometabolous insect *Manduca sexta*. This precaution was taken since such fragments could be present in standard double-distilled water. At various times post-immunization, animals were injected with an LD_{100} dose (5×10^5 CFU/g of body weight) of viable *P. aeruginosa*. For each trial, naive animals were also challenged to verify the accuracy of the LD_{100} dose, and bacteria were plated on TSA to obtain actual counts. Survival was scored 48 hr post-challenge.

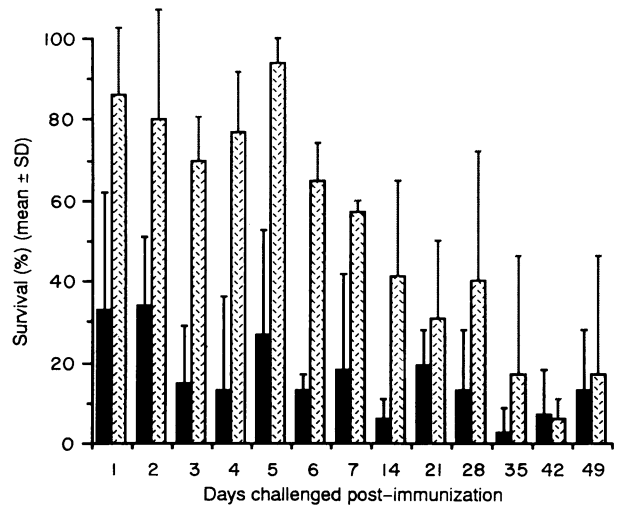

Figure 1. Kinetics of the antibacterial response. Adult male cockroaches were immunized with killed *Pseudomonas aeruginosa* (□) or injected with Burns–Tracey saline (■). Individual groups were challenged with viable *P. aeruginosa* at the times indicated post-immunization. Survival was scored 48 hr post-challenge. There were at least three trials per time-point.

Statistical analysis

The mean percentage survival was determined from at least three trials per time-period per immunizing organism. Each group consisted of 9–11 animals. Statistical significance was determined by the Mann–Whitney test.[9]

RESULTS

Animals were injected with either *P. aeruginosa* or BTS, rested for various periods of time before receiving a lethal challenge of bacteria, and the number of survivors recorded 48 hr post-challenge. Immunization with killed *P. aeruginosa* cells induced greater protection than injecting BTS, as evidenced by a significant increase ($P = 0·025$; Table 1) in mean percentage survival after challenge (Fig. 1). This response was demon-

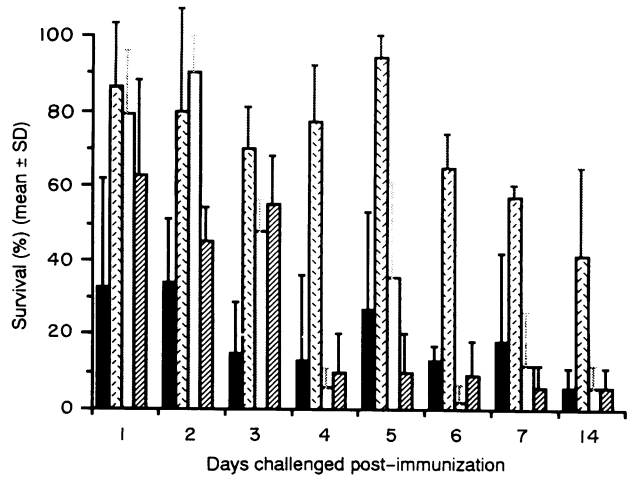

Figure 2. Specificity of the antibacterial response as determined by immunizing with Gram-positive bacteria. Adult male cockroaches were immunized with either fixed *Pseudomonas aeruginosa* (▨), *Micrococcus lysodeikticus* (□), *Streptococcus lactis* (▩) or injected with BTS (■). Individual groups were challenged with viable *P. aeruginosa* at the times indicated post-immunization. Survival was scored 48 hr post-challenge. There were at least three trials per time-point.

strated as early as Day 1 p.i., with protection not declining until after 14 days p.i. Animals were still protected against challenge as long as 21 and 28 days after immunization with *P. aeruginosa*, although the significance of the increase in survival relative to controls was borderline ($P=0{\cdot}1$). By day 35 p.i., there was no significant difference in survival between immune and control groups (Fig. 1).

To test the specificity of the response, animals were immunized with glutaraldehyde-fixed cells from several genera of bacteria and challenged with an LD_{100} dose of *P. aeruginosa* (a Gram-negative bacterium). Groups received injections of the Gram-positive organisms *Micrococcus lysodeikticus* or *Streptococcus lactis*, and were rested for various times before challenge. The mean percentage survival after challenge was measured for groups injected with either fixed Gram-positive bacteria, *P. aeruginosa* or BTS (Fig. 2). Immunization with *M. lysodeikticus* or *P. aeruginosa* stimulated similar protection when the challenge dose of *P. aeruginosa* was received within the first 2 days p.i. (Fig. 2, Table 2). The response generated by immunization with *M. lysodeikticus* provided less protection against challenge by Day 3 p.i. than immunization with *P. aeruginosa* for the same rest period. The mean percentage survival for this group was significantly lower ($P=0{\cdot}025$, Table 2) than the survival observed in the group immunized with *P. aeruginosa*, yet significantly higher than controls ($P=0{\cdot}025$; Table 1). At every time point (p.i.) tested, immunization with *S. lactis* induced less protection than immunization with *P. aeruginosa* (Fig. 2), as reflected by a significantly lower ($P\leqslant 0{\cdot}05$; Table 2) mean percentage survival. However, protection was also elicited by immunization with *S. lactis*. This organism induced a significantly higher ($P\leqslant 0{\cdot}1$; Table 1) survival rate than control groups injected with BTS when the animals were challenged within the first 3 days p.i. After Day 3 p.i., there was no significant difference in survival between groups injected with *S. lactis*, *M. lysodeikticus* or BTS (Table 1).

Table 2. Comparative specificity of the antibacterial response: significance of immunizing with various bacteria relative to immunizing with *Pseudomonas aeruginosa*

Days post-immunization	*E. cloacae*	*S. marescens*	*M. lysodeikticus*	*S. lactis*
1	$>0{\cdot}1$	$>0{\cdot}1$	$>0{\cdot}1$	0·05
2	$>0{\cdot}1$	$>0{\cdot}1$	$>0{\cdot}1$	0·05
3	$>0{\cdot}1$	$>0{\cdot}1$	0·025	0·05
4	0·025	0·025	0·025	0·025
5	0·025	0·025	0·025	0·025
6	$0{\cdot}05<P<0{\cdot}1$	0·05	0·025	0·025
7	0·025	0·025	0·025	0·025
14	$>0{\cdot}1$	0·025	0·025	0·025

The *P*-value for the difference in mean percentage survival after challenge is given for each group relative to the mean percentage survival resulting from immunization with *P. aeruginosa*. Days post-immunization (p.i.) refers to the day challenged p.i.

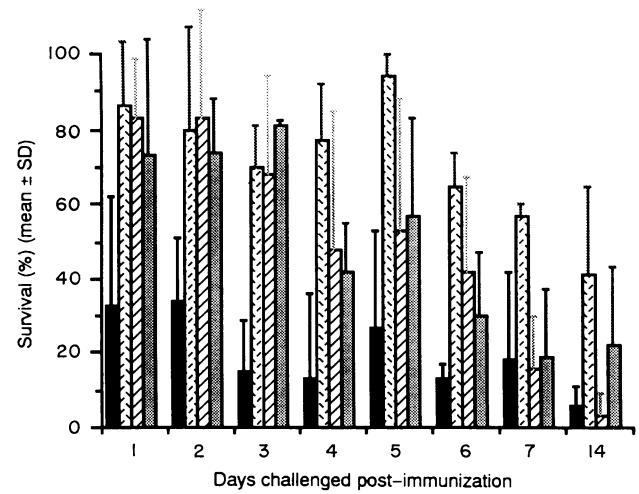

Figure 3. Specificity of the antibacterial response as determined by immunizing with Gram-negative bacteria. Adult male cockroaches were immunized with fixed *Pseudomonas aeruginosa* (▨), *Serratia marcescens* (▩), *Enterobacter cloacae* (▩) or injected with BTS (■). Individual groups were challenged with viable *P. aeruginosa* at the times indicated post-immunization. Survival was scored 48 hr post-challenge. There were at least three trials per time-point.

The data presented in Fig. 2 indicate that the response induced by bacteria developed selectively, distinguishing between Gram-positive and Gram-negative organisms at the longer times post-immunization. To determine whether the ability to specifically recognize the immunizing organism was sophisticated enough to distinguish between Gram-negative genera, animals were immunized with either *Pseudomonas aeruginosa, Enterobacter cloacae* or *Serratia marcescens* and challenged with *P. aeruginosa*. Figure 3 shows the kinetics of the response stimulated by injection of Gram-negative bacteria or BTS. When the challenge dose was received within the first 3 days after immunization, there was no significant difference in

survival between the three groups (Table 2). By Day 4 after immunization with either *E. cloacae* or *S. marcescens*, survival was similar to that of control groups (Table 1), and significantly lower ($P=0{\cdot}025$; Table 2) than that of animals immunized with *P. aeruginosa*. When the challenge dose was received 5 days p.i., there was significantly more protection induced by immunizing with *P. aeruginosa* than the other Gram-negative bacteria, although immunizing with *E. cloacae* or *S. marcescens* still resulted in significant survival rates as compared with BTS controls (Table 1). By Day 6 p.i., *S. marcescens* protection against challenge was still significantly higher than controls, but protection was no longer evident by Day 7 p.i. (Table 1).

DISCUSSION

The antibacterial response of the adult male cockroach appears to be composed of two phases: the first phase resembles a typical holometabolous antibacterial response, while the second phase is similar to the response elicited by soluble protein antigens in adult roaches. The initial non-specific phase was short-lived, displaying activity as early as 24 hr p.i., but then apparently declining by 72 hr p.i. The acute phase was characterized by being non-specific in nature, since *M. lysodeikticus*, *S. lactis*, *E. cloacae* and *S. marcescens* all generated significant protection against *P. aeruginosa* challenge as compared with BTS controls ($P\geq 0{\cdot}1$, where $P=0{\cdot}1$ is considered of borderline significance; Table 1). In fact, *M. lysodeikticus*, *E. cloacae* and *S. marcescens* induced protection similar to that induced by *P. aeruginosa* itself ($P>0{\cdot}1$; Table 2). Thus, even when the immunizing organism (i.e. *S. lactis*) did not elicit as significant a response as immunizing with *P. aeruginosa*, some degree of non-specific protection was induced and lasted until 3 days p.i. By Day 4 p.i., the non-specific protection subsided (Table 2). Although protection induced by immunizing with bacteria was significant compared with BTS controls, some protection was induced by BTS (Fig. 1). Dunn[1] has demonstrated that pupae maintained under sterile conditions do not respond to injections of sterile saline. The roaches used in these studies were not axenic, so during immunization exogenous bacteria may have been inadvertently introduced, thus inducing a minimal response.

The second phase of the response which more closely resembled true immunity then emerged. This phase was characterized by a high degree of specificity, as evidenced by the significantly greater protection against challenge elicited by immunizing with *P. aeruginosa* as compared with immunizing with other Gram-positive or Gram-negative bacteria tested. Some protection was observed at Day 5 p.i. with Gram-negative bacteria (and Day 6 p.i. with *S. marcescens*) compared with BTS controls (Table 1). This relatively specific protection (generated only by Gram-negative bacteria) may have been the beginning of the second phase, which required a longer period after immunization (7 days) to develop its highly selective nature. A significant response was observed with *E. cloacae* at Day 14 p.i. compared with BTS controls. We cannot explain this response other than to suggest that these animals may have been stimulated by bacteria present in their environment. The length of the antibacterial response (28 days p.i.), and the ability to specifically recognize the immunizing bacterium were similar to the characteristics of the humoral immune response induced by soluble protein antigens in the adult cockroach.[5] Thus, the antibacterial response of the adult male cockroach possesses characteristics of both the holometabolous antibacterial response as well as an adaptive immune response. The roach appears to utilize a quick, non-selective mechanism to combat the initial infection in the haemocoel, while the sustained, highly specific mechanism continues to protect against any remaining bacteria.

Further studies will be directed at defining the mechanisms (i.e. cellular and/or humoral) mediating the antibacterial response of the roach. The determination of these mechanisms, and how they relate to those mediating the holometabolous antibacterial response or the adaptive immune response to soluble proteins in the cockroach, will provide some new insights into the extent of insect immunity.

ACKNOWLEDGMENTS

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