Exposure to *Schistosoma mansoni* antigen induces an allergic response to peanuts in an American cockroach model

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SUMMARY

Across the world, tens of millions of people suffer from peanut allergies. Despite the large amount of research conducted on the topic, the root cause of this disease is still unknown. Peanut allergy is characterized by an immune hypersensitivity reaction to a peanut antigen, which is bound by immunoglobulin E (IgE), the antibody associated with parasitic infections as well as allergies. Previous research has shown that a protein, Ara-h-1, found in peanuts, is cross-reactive with the IPSE/α-1 and κ-5 proteins found in the eggs of the parasitic blood fluke, *Schistosoma mansoni.* Here, we propose that this cross-reactivity contributes to the development of peanut allergies. We investigated the relationship between *S. mansoni* and peanut allergy using an American cockroach model, which was selected due to its capability to exhibit an immune response with both memory and specificity, making its humoral immunity comparable to that of humans. Based on our observations, we established that cockroaches injected with a soluble *S. mansoni* egg antigen exhibited physical symptoms of an allergic reaction after consuming peanuts, likely due to the aforementioned cross-reactivity. This research demonstrates a novel, direct association between a parasitic worm and food allergies *in vivo*. These results have the potential to change the way the scientific community views peanut allergies, as well as other food allergies, and open new avenues of research with the hope of finding a cure.

INTRODUCTION

Peanut allergy is a potentially life-threatening condition that affects approximately 70 million people worldwide. Despite the prevalence of this allergy, its cause is currently unknown. There are many differing hypotheses regarding the cause of food allergy. One of the most mainstream is the notion that allergies developed as misdirected immune responses to substances that the immune system would normally recognize as innocuous (1).

Peanut allergy consists of an immune hypersensitivity reaction that is triggered by the binding of a peanut antigen, a molecule that elicits an immune response, to a specific antibody, a protein that identifies and neutralizes antigens (1-3). A peanut allergy begins when an individual comes into contact with said antigen through one of three ways: the respiratory mucous membrane, called the mucosa, the gastrointestinal mucosa, or the skin. Upon contact, small particles of the antigen diffuse into the mucosa or skin and are detected by immune cells located in epithelial membranes, such as the skin and the lining of the digestive tract, which trigger a Type 2 immune response (1, 3). This type of immune response is associated with allergic reactions, as well as parasitic worm infections, in part through the production of Immunoglobulin E (IgE) antibodies (1, 4). When an allergen is initially detected near epithelial membranes by antigen-presenting immune cells, it binds to specialized receptors on these cells. This triggers a series of reactions, eventually causing immune cells, called B-cells, to generate immunological memory and produce IgE antibodies specific to the allergen. This process is called sensitization and primes the immune system to quickly react upon subsequent exposure to the same allergen (1).

When the allergen is detected again, B cells quickly produce antigen-specific IgE antibodies, which activate another series of reactions including the release of specific signaling compounds (1). In response to the signaling compounds, many somatic cells undergo changes; vascular and epithelial cells become more permeable, epithelial cells produce more mucus, and smooth muscle cells become more contractile. These changes to non-immune cells result in the outward symptoms of an allergic reaction such as swelling, a rash, hives, constriction of the airway, and constriction of the gastrointestinal tract, which can lead to vomiting (1, 3). On occasion, there may be two phases of an allergic reaction. The initial reaction is caused by the release of histamine, and the late-phase reaction is caused by the recruitment of additional immune cells involved in Type 2 immune responses. Both reactions have similar symptoms, though late-phase reactions may result in long-term inflammation (1).

The parasitic worm *Schistosoma mansoni* is a species of blood trematode that is one of the leading causes of the disease schistosomiasis in humans. Currently, there are over 200 million people infected with schistosomes, making this the second most devastating parasitic disease after malaria. *S. mansoni*'s life cycle is complex, involving numerous larval stages and an intermediate host before maturation in the human liver (**Figure 1**) (5). One of the most notable features of *S. mansoni* is its immunosuppressive ability. Many parasitic worms, or helminths, possess such abilities, however the ability of *S. mansoni* is extremely potent because it allows for

the easy excretion of eggs, which is integral to the parasite's life cycle. When a female *S. mansoni* lays eggs, the eggs travel from the host's blood vessels into their intestines, which leaves small channels that allow bacteria, viruses, and other microscopic organisms to enter the gut wall. Approximately 150 eggs make this journey every day, which leads to numerous small channels being formed. Ordinarily, this would result in large-scale inflammation; however, *S. mansoni* eggs secrete a protein called **I**nterleukin-4-inducing **p**rinciple of **s**chistosome **e**ggs (IPSE)/α-1, which suppresses the immune system's natural inflammatory response (6). In addition, adult *S. mansoni* excrete prostaglandins and other immunosuppressant molecules, which limit the ability of the host's immune system to defend against the helminth. Currently, there are three major proteins found in *S. mansoni* eggs that have been identified as antigens and are the targets of the host Type 2 immune response: IPSE/α-1, κ-5, and ω-1 (7, 8). This indicates that IPSE/α-1 is both an immunosuppressive molecule and a target of the host immune response against *S. mansoni*. When these proteins are detected in the body, they are bound by IgE, which signals their presence and triggers an immune response.

Due to the similarities between the immune responses involved in parasitic infections and allergies, it is thought that IgE-mediated immune responses evolved to defend the body against parasites, rather than to cause allergies (9). Along these lines, previous research has suggested a possible connection between parasites and allergies by finding similarities between the sequences and structures of proteins found in common parasites and allergens (9). A similar study focused on the three known antigens in *S. mansoni* eggs, and found that IPSE/α-1 and κ-5 were cross-reactive with the peanut antigen Ara-h-1 (10). This cross-reactivity occurs due to similarities between these antigen molecules, causing antibodies that primarily target the *S. mansoni* egg antigens to also target Ara-h-1, though the nature of these similarities is currently unknown (10). In another study, it was discovered that many peanut antigens are cross-reactive with one another (11). Thus, the more types of cross-reactive antigens present, the more likely an allergic individual is to experience a severe reaction (11). Additionally, *S. mansoni* egg antigens have been found to be cross-reactive with other antigens, such as latex and birch pollen (10, 12). These allergens are also cross-reactive with peanuts (13, 14). The relationship between peanut and other allergens that are cross-reactive with *S. mansoni* has been demonstrated on a clinical level, since individuals with peanut allergies are commonly found to be allergic to latex and birch pollen as well (14). These examples of *S. mansoni*'s cross-reactivity suggests that *S. mansoni*-specific antibodies could play a role in the development of peanut allergy.

In this study, we used an American cockroach (*Periplaneta americana*) model to investigate the association between *S. mansoni* and peanut allergy. A notable feature of this insect is that, like mammals, it can mount a functional adaptive immune
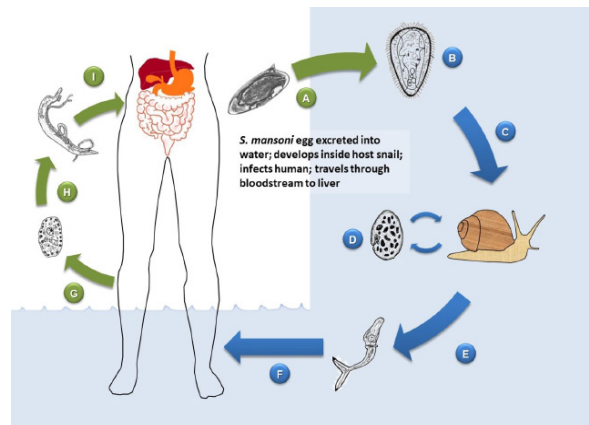
Figure 1. Life Cycle of *S. mansoni*. A) Infected humans defecate into a water source, releasing *S. mansoni* eggs into the water. **B)** The eggs hatch and release a larval stage called miracidia. **C)** The miracidia seek out and infect a primary host, the snail *Biomphalaria glabrata*. **D)** In the tissue of *B. glabrata*, the miracidia develop into another larval stage, sporocysts. **E)** The sporocysts reproduce inside the snail, and the successive generation develops into the larval stage cercariae, which are released from the snail into the body of water. **F)** The free-swimming cercariae infect human hosts by penetrating the skin and entering the blood vessel when a human steps into the water. **G)** Upon infection, the cercariae lose their tails and develop into schistosomulae. **H)** The schistosomulae migrate to the liver through the bloodstream, where they mature into adults. **I)** The adult *S. mansoni* lay eggs, which circulate to the liver, as well as the intestines, where the eggs migrate through the gut wall and into fecal matter.

response to antigens. Structural and functional parallels can be drawn between mammalian antibodies and certain proteins involved in insect humoral immune responses, suggesting that many of the principles of mammalian immunity may also apply to cockroaches. Additionally, some insects, including cockroaches, produce hemocytes and immunocytes, which are the equivalent of mammalian red and white blood cells, respectively, and allow these insects to have an immunological memory that is functionally analogous to that of mammals (15). American cockroaches have a long-lasting, specific, humoral response to soluble antigens, much like vertebrates do (16). A protein complex that is thought to have a similar function to mammalian antibodies has been identified in the American cockroach, though the similarity between this protein complex and mammalian antibodies is currently unknown (17).

Through this study, we sought to determine if the presence of *S. mansoni* in the body leads to peanut allergies *in vivo*. We observed that injecting cockroaches with a prepared *S. mansoni* soluble egg antigen (SmSEA), containing the three immunogenic proteins found in *S. mansoni* eggs, led to the cockroaches exhibiting physical symptoms of an allergic reaction after consuming peanuts, likely due to the cross-reactivity between the *S. mansoni* antigens and peanut antigens. Based on our results and a synthesis of previously-published research, we also propose

a pathway of development of some peanut allergy cases, where maternal infection with *S. mansoni* could determine if a child is allergic to peanuts. This research demonstrates a novel, direct association between a parasitic worm and food allergies in vivo and has the potential to open new avenues of research on food allergy with the hope of finding a cure.

RESULTS

Confirmation Phase

We conducted this study in two phases, the first being a confirmation phase to determine if injection with SmSEA resulted in an immune response, as established by previous research (**Figure 2**) (18). In the Confirmation Phase, we established two groups of cockroaches and designated them as the Naïve Group and the SmSEA Group. We injected the SmSEA Group with 5 µg of SmSEA twice with a one-week interval between each injection at a concentration of 0.5 mg/mL, and we did not inject the Naïve Group with any solution. We video recorded the reactions of the cockroaches for eight hours post-injection to look for signs of an immune response (3). We then assigned each symptom of an immune response to a reaction score and scored the reactions of the cockroaches in each group according to their severity (**Table 1**).

We did not observe cockroaches in the Naïve Group exhibiting symptoms of an immune response, so we used their reaction as a baseline of comparison for all other cockroach groups (**Figure 3A**). Cockroaches in the SmSEA Group clearly displayed symptoms of an immune response, as every cockroach had a reaction score of 1 or greater, and 27.3% of the cockroaches died within 72 hours of the first injection (**Figure 3B**). We expected this severe reaction to occur because SmSEA is known to induce an extremely strong immune reaction in most species (18). Additionally, the surviving cockroaches exhibited symptoms of a less severe immune response after their second injection with SmSEA, as all cockroaches had a reaction score of 1. This decrease in the severity of cockroach immune reactions has been observed in previous research using various antigens and is a characteristic of humoral immunity (17). The first injection primed the cockroaches' immune systems to defend against subsequent exposures to SmSEA. Thus, the cockroaches developed a level of protection against SmSEA, causing them to exhibit symptoms of a less severe immune reaction after their second injection.

Experimental Phase

The second phase of this research was an experimental phase to determine if the introduction of SmSEA induced an allergic reaction to peanuts. We established two additional groups of cockroaches and designated them as the Peanut Group and the SmSEA+Peanut Group. We injected the SmSEA+Peanut Group cockroaches with the determined 5 µg of SmSEA twice with a one-week interval between each injection and video recorded the reactions of the cockroaches
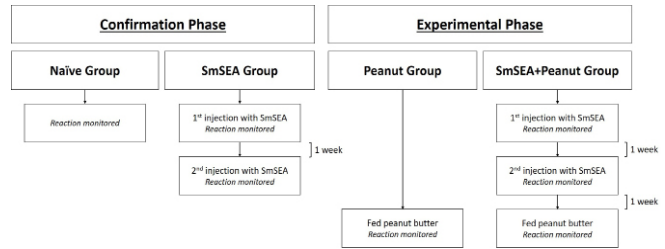
Figure 2. Diagram of Experimental Design. Under the Confirmation Phase, two groups of cockroaches were established: the Naïve Group and the SmSEA Group. The Naïve Group was not given any treatment, while the SmSEA Group was injected with SmSEA twice with a one-week interval between injections. Under the Experimental Phase, two more groups of cockroaches were established: the Peanut Group and the SmSEA+Peanut Group. The Peanut Group was fed pure peanut butter, while the SmSEA+Peanut Group was injected with SmSEA twice with a one-week interval between injections, and then fed peanut butter.

Reaction Score	Symptoms
0	No symptoms
1	Slow movements; motionless for short periods (< 5 min); self-isolation
2	Motionless for long periods (> 5 min)
3	Convulsions; tremors
4	Death

Table 1. Reaction Scores Assigned to Cockroaches. The symptoms exhibited by cockroaches during the monitoring period were ranked on a scale from 0 – 4, with 0 being no visible symptoms of an immune response and 4 being death. The recorded reaction score was the highest score each cockroach exhibited during the monitoring period. For example, a cockroach exhibiting a symptom score of 3 was understood to have exhibited symptom scores of 1 and 2 as well.
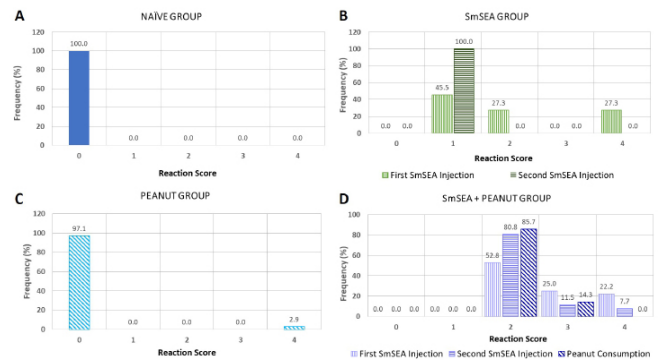
Figure 3. Reaction scores of cockroach groups during the eight-hour monitoring period. Reactions during the monitoring period of **A)** the Naïve Group, **B)** the SmSEA Group after the first and second injections, **C)** the Peanut Group after consuming peanuts, and **D)** the SmSEA+Peanut Group after the first injection, second injection, and peanut consumption.

for eight hours post-injection. We did not inject the Peanut Group with any solution. After one more week, we provided pure peanut butter to both groups and video recorded their reactions for eight hours to monitor them for symptoms of an allergic reaction. We classified immune responses to peanut butter as an allergic reaction because cockroaches are commonly known to feed on peanut butter, so an immune

response to it constitutes the definition of an allergic reaction (19).

As expected, most of the cockroaches in the Peanut Group displayed no symptoms of an allergic reaction to peanut butter and had reaction scores of zero (**Figure 3C**). Similar to the SmSEA Group, cockroaches in the SmSEA+Peanut Group also exhibited severe symptoms of an immune response after injection with SmSEA, with several of them dying within 72 hours of injection. Additionally, cockroaches exhibited symptoms of a less severe immune response after their second injection. More notably, the SmSEA+Peanut Group cockroaches exhibited a moderate allergic reaction to peanut butter, with no reaction score less than 2, but without any fatalities (**Figure 3D**).

Analysis

By comparing the reaction scores across groups, we established that the Naïve Group and the Peanut Group had little to no symptoms of an immune reaction, the SmSEA Group displayed symptoms of a severe immune response after injection with SmSEA, and the SmSEA+Peanut Group displayed symptoms of a moderate allergic reaction after consuming peanut butter (**Figure 4**).

To add further evidence to the conclusions drawn from the visual representation of our data, we conducted a one-tailed Kruskal-Wallis H-test, where we discovered that the distribution of reaction scores was not uniform across all groups (H-test, $p < 0.00001$), indicating that at least one cockroach group experienced a significant immune response to an antigen. Then, we performed a number of one-tailed post hoc Mann-Whitney U-tests and used a Benjamini-Hochberg correction to account for Type I error due to multiple comparisons.

Comparing the reactions of the Naïve Group and the Peanut Group showed that there was no significant difference between the reactions of naïve cockroaches and those provided with pure peanut butter (U-test, $p = 0.448$), as anticipated. We can conclude that exposure of cockroaches to peanuts did not result in an immune response. This result served as a baseline of comparison.

Cockroaches in the SmSEA Group exhibited significantly higher reaction scores than those in the Naïve Group (U-test, $p < 0.00001$). This indicated that cockroaches injected with SmSEA experienced a significant immune response. For the SmSEA Group, we combined data from the first and second SmSEA injections into a single group to accurately represent all immune responses for the purpose of these statistical tests. We categorized the reaction to SmSEA as "strong" because cockroaches exhibited symptoms of a severe immune response and even died after injection with SmSEA. This result corroborates previous research, which has shown that SmSEA induces an extremely strong immune reaction in most species (18).

There was a significant difference between the reactions
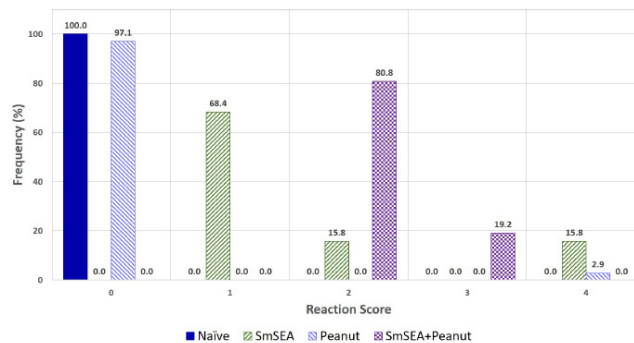
Figure 4. Cockroaches in the SmSEA and SmSEA+Peanut Groups exhibited higher reaction scores than cockroaches in the Naïve and Peanut Groups. Here, the data from the SmSEA Group were combined into a single group, so there is no distinction between the reactions to the first and second injections. The SmSEA+Peanut Group graph represents the cockroaches' reactions after consuming peanuts.

of cockroaches in the Naïve Group and those in the SmSEA+Peanut Group, which consumed peanut butter after injection with SmSEA (U-test, $p < 0.00001$). This indicates that cockroaches developed an allergy to peanuts after injection with SmSEA. For the SmSEA+Peanut Group, we exclusively used the data from the cockroaches' reactions after consuming peanut butter for these tests to accurately represent their allergic reactions to peanuts.

A comparison between the reactions of the Peanut Group and the SmSEA+Peanut Group revealed that cockroaches injected with SmSEA have a more severe allergic response to peanuts than cockroaches not injected with SmSEA (U-test, $p < 0.00001$). This result signifies that it was not the consumption of peanut butter that led to the development of the allergic response, but rather the injection with SmSEA.

The immune response exhibited by the SmSEA Group was more severe than the allergic response exhibited by the SmSEA+Peanut Group (U-test, $p = 0.00145$). We anticipated this result, as we hypothesized that the immune response to peanuts was caused by the cross-reactivity between peanut and *S. mansoni* antigens. This may have caused the reaction to the primary immune target, the SmSEA, to be stronger than the reaction to peanut butter. Thus, we categorized the SmSEA+Peanut Group's reaction to peanuts as "moderate."

DISCUSSION

With the goal of furthering research on the cause of peanut allergy, we injected American cockroaches with SmSEA before feeding them peanut butter. Our results established that cockroaches that were injected with SmSEA and then fed peanuts experienced an allergic reaction to peanuts. Additionally, we also showed that cockroaches injected with SmSEA had a greater immune response than the allergic reaction of cockroaches that were injected with SmSEA and fed peanuts. This demonstrates that cockroaches developed

a moderate allergic reaction to peanuts after being injected with SmSEA.

Here, we propose a possible pathway of development of some peanut allergy cases. If a child is born to a mother who was previously exposed to *S. mansoni*, then the child will receive components of their mother's immunity against the blood fluke through passive transfer, including anti-*S. mansoni* IgE, as well as the *S. mansoni* soluble egg antigens circulating in her bloodstream (20, 21). Due to immunosuppression by certain *S. mansoni* soluble egg antigens, the child's immune system will not mount a widespread, systemic response against these antigens (6). However, the child's immune system will still be sensitized to, and produce antibodies against, the soluble egg antigens because the immunosuppression by the antigens alone is much milder than that of *S. mansoni*, allowing for greater immune function (6, 21, 22). If the child then comes into contact with a peanut, their immune system will mount a response against the peanut antigens, due to the lack of immunosuppression by peanuts and the cross-reactivity of *S. mansoni* soluble egg antigens with certain peanut proteins. This cross-reactivity between peanut and *S. mansoni* antigens is what we propose leads to an allergic reaction.

Our results provide initial support for our proposed pathway of peanut allergy development by demonstrating that the cross-reactivity of SmSEA with peanuts contributes to the development of a peanut allergy. The cockroaches that we injected with SmSEA were a model for a child born to a mother with immunity to *S. mansoni*; both have *S. mansoni* soluble egg antigens being transported around their body via the circulatory system. Therefore, when the cockroaches in the SmSEA+Peanut Group exhibited an allergic reaction after consuming peanut butter, we likened their reaction to that of a child with a developed immune system eating a peanut and experiencing an allergic reaction. We believe that the aforementioned cross-reactivity could play a role in the development of peanut allergies in some children. However, further research is needed to provide more evidence that vertically transferred immunity to *S. mansoni* results in a peanut allergy.

As with the majority of studies, this study is subject to limitations. A factor that potentially impacts the results is observer bias; however, measures were taken to mitigate the effect of this bias, such as video recording each trial during the 8-hour window in which a possible immune reaction would occur, defining time intervals for motionlessness which indicated a reaction score of 1 or 2, and setting a defined duration for each trial. Another limitation of this study was using an American cockroach model instead of the more commonly used murine model. This model was chosen due to our institutional constraints on conducting research using vertebrates; however, due to the similarities between the cockroach and human immune systems, the American cockroach is still a viable model organism. A final limitation was the design of the Naïve Group, which was not injected with any solution and hence, did not control for the effect of injection with a solution on the cockroach immune response. Further research may be conducted to investigate this effect. However, this limitation does not negate our observed results, as the SmSEA+Peanut Group experienced an allergic reaction to peanuts directly following the oral consumption of peanut butter. Additionally, previous studies regarding the immune response of cockroaches have established that injection with a saline solution as a negative control evokes a mild to negligible immune response in cockroaches (17). Since the SmSEA was prepared with saline, the cockroaches' immune reactions to SmSEA were most likely due to the antigen itself, as opposed to injection with the solution.

In this paper, we demonstrate that injection with SmSEA leads to an allergic reaction to peanuts in an American cockroach model. This study demonstrated a novel, direct association between a parasitic worm and a food allergy *in vivo*. The scope of this research was to study physical symptoms of an allergic reaction, and future research should be conducted to investigate if antibodies from cockroaches that are treated as those in this study exhibit cross-reactivity between *S. mansoni* egg antigens and peanut antigens. This could provide further support for our theory and minimize the possibility of alternative explanations for the observations in this study. Similarly, studying the intergenerational aspect of our proposed pathway of peanut allergy development would provide more insight into the potentially vertically transmissible nature of allergy. The outcome of our study can have a significant impact on how the scientific community views peanut and other food allergies, hopefully leading to further research and eventually improvement in the quality of life for millions worldwide.

MATERIALS AND METHODS
Organism Maintenance

Female cockroaches were obtained from Carolina Biological Supply, maintained in 43 L clear plastic bins with lids, and separate bins were established for each experimental group of cockroaches. We chose to use female cockroaches because they have been shown to produce a stronger immune response than male cockroaches and reach their peak immune response in one week, as opposed to males' two weeks (16, 17). A small container with hydrated water polymer crystals – to keep the cockroaches hydrated and prevent them from drowning – and a three-centimeter cube of potato as food was placed in each bin and replaced as needed. An eight-centimeter wide band of petroleum jelly was applied around the mouth of the bin to prevent escape, and it was reapplied every two months. The bins were kept at 24°C with a reptile heat mat, and each bin was misted with water daily to maintain a humidity of approximately 70%. A series of small holes were drilled into the lids to allow the oxygen concentration to remain high in the bin, and every two weeks, the bins were cleaned. Cleaning the bins entailed anesthetizing the cockroaches by submerging each bin in an ice water bath until the temperature inside the box was

recorded as 5.5°C and wiping the inside of the bin down with a damp cloth. Any egg cases or juvenile cockroaches present were disposed of by first freezing them using electronic anti-static freezing spray and then disposing of them in the garbage (23, 24).

Confirmation Phase

Two groups of cockroaches, the SmSEA Group (*n* = 11) and the Naïve Group (*n* = 10), were established. The SmSEA Group cockroaches were anesthetized by submerging a box containing cockroaches in ice water until the internal temperature of the box was 5.5°C. SmSEA was prepared according to protocols established by the Schistosomiasis Resource Center (SRC), part of the Biomedical Resource Institute at the National Institutes of Health, and obtained through a collaboration with the SRC for this research (25). Each cockroach was injected with 10 μL of SmSEA, diluted to 0.5 mg/mL with phosphate buffered saline (PBS), between its 4th and 5th abdominal sternites using a 50 μL Hamilton syringe. By a mass ratio, this concentration of SmSEA has been shown to induce a non-lethal immune response in mice; therefore, we used it for our concentration in this study (18). After injection, the cockroach was placed back into its bin, and this process was repeated for the remaining cockroaches in its group. The Naïve Group was also submerged in ice water for the requisite amount of time but was not injected with any solution. A video camera was set up above the bins of each group, and the cockroaches' reactions were recorded for eight hours post-injection in order to record any adverse reactions. Previous research using a murine model established that if any allergic reaction were to occur, it would occur within eight hours of exposure to an allergen (3). After seven days, this injection procedure was repeated with the remaining cockroaches (Naïve Group: *n* = 10; SmSEA Group: *n* = 8) to increase the cockroaches' immune reactions (17). The severity of the cockroaches' reactions was ranked on a scale from 0-4 (**Table 1**) (26).

Experimental Phase

Two groups of cockroaches, the SmSEA+Peanut Group (*n* = 36) and the Peanut Group (*n* = 34) were established. The injection procedure described above for the SmSEA Group was used for the SmSEA+Peanut Group, while the Peanut Group was not injected with any solution. Five days after the second SmSEA injection, (Peanut Group: *n* = 34; SmSEA+Peanut Group: *n* = 27) the cube of potato was removed from both of the cockroach groups' bins to ensure the cockroaches would later eat the peanut butter provided during the trial. Two days later, 15 mL of organic, no-salt-added peanut butter (Nature's Promise) was placed in a small plastic container inside each bin. A video camera was set up above each the bins of each group, and the cockroaches' reactions were video recorded for eight hours post-administration. The severity of the cockroaches' reactions was again ranked on a scale from 0-4.

Video Analysis

The video recording from each trial was played back, and each instance when a cockroach exhibited a symptom of an allergic reaction was noted. The highest reaction score that each cockroach exhibited over the course of the trial was noted, and the total number of cockroaches for each reaction score was recorded. For example, if a cockroach exhibited reaction scores of 1, 2, and 3 as their immune response intensified, the highest score of 3 was be recorded for the cockroach.

Statistical Analysis

To be able to accurately compare results across groups, we expressed the frequency of observed reaction scores as a percentage of the total. Using Microsoft Excel and Fathom Dynamic Data Software, we performed a Kruskal-Wallis H-Test (α = 0.05) to initially establish the difference in reaction scores between all cockroach groups. Then, we used Mann-Whitney U-Tests (α = 0.05) with a Benjamini-Hochberg Correction (*q* = 0.00415) post hoc to compare each group to every other group and establish the difference in reaction scores between each group. We chose non-parametric statistical tests because we did not have the required number of data points to establish a normal distribution, which is a condition of parametric statistical analysis.

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