



The perils and prospects of using phytohaemagglutinin in evolutionary ecology

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Several techniques are available for quantifying the vertebrate immune response, information that is particularly useful for understanding the contribution of immunity to the evolution of life-history strategies. The most widely used is the phytohaemagglutinin (PHA) skin-swelling technique, which is usually regarded as an index of acquired immunity. However, our understanding of the effects of PHA in skin is poor, despite the fact that it has implications for what the test can tell us about immune activity. As we discuss here, a recent study by Martin and colleagues on the response to PHA at the cellular level in wild birds has highlighted the relative extent to which PHA-induced swelling, as most commonly applied, measures innate immunity versus acquired immunity.

Immunoecology

Over the past decade, there has been increasing interest in parasite–host interactions, which can affect life-history evolution, sexual selection strategies and the population dynamics of the host [1]. The ability of a host to prevent and control infection by pathogens (i.e. its immunocompetence) might influence tradeoffs between life-history traits (e.g. reproductive effort or sexual ornamentation) and the immune system itself [1,2]. Vertebrates invest substantial resources in immune systems, particularly during an infection episode, so it is crucial to understand the contribution of immunity to the evolution of life-history strategies.

Ecologists have made progress in exploring tradeoffs between immunological variables and life-history components by using techniques that measure a single indicator of immunocompetence, mainly in birds [2]. However, we still do not know fully how the immunological measurements inform us about the immune system in wild organisms: specifically, whether they provide a measure of acquired versus innate immune reactions. How these measures relate proportionately to the unmeasured components of the immune system has received some discussion, but is still poorly understood [2]. Here we discuss potential problems in the interpretation of results derived from the most commonly used measure of immunity in birds, the phytohaemagglutinin (PHA) skin test, drawing on established immunological phenomena and a recent investigation of the PHA response at the cellular level in wild birds by Martin and colleagues [3].

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Ecological immunology techniques

The vertebrate immune system comprises an integrated system of non-specific (innate) and pathogen-specific memory (acquired) responses, which are triggered by tissue trauma and infection (Box 1). The first immune measures applied by vertebrate ecologists were techniques to monitor factors such as leukocyte counts, and concentrations of immunoglobulins and other plasma proteins. It is unclear, however, whether high levels of a given factor reflect the status of current infections, high immunocompetence, or the opposite, that is, inefficient immunity to infections such that an individual needs to exist in a high state of activation [2]. This problem prompted ecologists to use challenge techniques instead, where the immune system is exposed to a novel stimulus and the resulting response quantified. Such a technique that is easy to use and which is popular with avian ecologists is the skin swelling elicited by injection of PHA; the swelling is assumed to be a measure of the T lymphocyte-mediated acquired immune system [4,5] (Box 1). This assumption, however, remained untested in wild birds until a recent study by Martin and colleagues [3].

In addition, PHA is generally considered to activate specifically T lymphocytes (which are either already present in low numbers at the site of injection or attracted there from the blood) to undergo mitosis, such that the size of the swelling induced by PHA is a measure of acquired, cell-mediated immunity. However, PHA not only induces T lymphocyte mitogenesis, but is also likely to cause local inflammation as a direct result of tissue damage (Box 1). Both processes are possible, and the relative strength of the mitogenic versus agglutinin activities of PHA depends on the presence of the specific cell-surface receptors to which PHA binds. Therefore, it is important to assess the specific effects of PHA in the study organism, given that the PHA-induced effects established in one taxonomic group might not be identical to those of other groups because of differences in their cell-surface receptors. Martin *et al.* included such a consideration in their recent paper on wild birds [3].

Does PHA trigger innate or acquired immune responses?

It is likely that PHA triggers both innate and acquired immune responses, but with different timings after a single or subsequent exposure. The expectation is that, by its nature as a foreign, toxic protein, PHA will first engender a reaction to tissue damage, followed by an adaptive response to it as a new antigen. One approach

Box 1. PHA and the immune defence system

The immune system in a nutshell

Infection by a pathogen or parasite triggers a suit of responses by the vertebrate immune system. Initially, innate responses are triggered by the detection of generic types of pathogen and tissue damage caused by the infection. These innate responses exhibit no memory and are induced at the same intensity upon contact with the same pathogen a second time. Such responses stimulate the development of acquired, pathogen-specific immunity that responds more rapidly and with higher affinity on subsequent exposure, invoking pathogen-specific antibody and T cell responses, and can therefore provide (hopefully) lifelong immunity and no disease on subsequent exposure.

What is PHA?

PHA is derived from red kidney beans *Phaseolus vulgaris* and belongs to a group of carbohydrate-binding proteins, lectins, which are common in plant seeds, where they are thought to act against herbivory (hence the need to cook red kidney beans at high temperature for human consumption). Lectins are proteins with two or more carbohydrate-binding sites and can crosslink and agglutinate cells bearing their target carbohydrates, which vary between cell types. As for many lectins, PHA was first identified by its ability to agglutinate blood cells, hence its name. It can also induce cells to enter mitosis, presumably by cross-linking cell-surface receptors that are related to cell activation. When purified mammalian lymphocytes are exposed to PHA, mitosis of T lymphocytes is preferentially stimulated. This has led to PHA being commonly used as a T cell mitogen in human and mouse immunology. A similar preferential stimulation of T lymphocytes is also thought to occur in chickens but, as in mammals, PHA might react with more than one cell type [4].

What does PHA do?

PHA is designed to be harmful to animal tissues [15]. The overall effect of injecting it into a complex, cell-type diverse tissue with a blood circulation, such as skin, will be a complex of cell activation, destruction and infiltration. The immediate response to this trauma will be a local inflammatory response encompassing increased blood supply and cellular infiltration into the damaged tissue. In mammals, the first cell type to arrive in inflammation is usually neutrophils, which are the equivalent of heterophils in birds. This is followed by other cell types, including those directly involved in antigen presentation to the acquired immune system for a memory B and T cell response to PHA as a foreign antigen. When the thickness of the swelling is usually measured (24 or 48 hours after a single exposure in birds), it is likely that only the first phase of the response is underway and infiltration by T cells is likely to be a relatively minor, but increasing, component.

to establishing the true nature of the PHA response in birds, however, is to examine the cell types that accumulate with time at the injection site. Martin and colleagues carried this out for the first time in wild birds [3], extending a much earlier study on domestic chickens [6]. They gave a single injection of PHA to house sparrows *Passer domesticus*, measured the swelling and examined the nature of the cellular infiltrate histologically over the following 48 hours. This revealed that PHA elicited cellular responses that were indicative of both innate and acquired responses with different time courses.

There was an intense infiltration of many cell types, with the relative abundance of each type peaking at different times. Heterophils and thrombocytes appeared first, followed by basophils and macrophages, whereas there was no clear peak for lymphocytes. Only a small proportion of all the correlations examined between abundance of a particular cell type and thickness of the swelling at different

times after the injection were statistically significant, and these also varied in direction. Significantly, swelling thickness only related to the abundance of lymphocytes six hours after injection and this only in a sub-set of the birds. The swelling after 24 hours, when most studies on birds measure the thickness, was inversely related to heterophil infiltration, these cells having peaked and predominated earlier. The results highlight the complex and dynamic nature of the short-term swelling response to PHA-injection, which clearly involves both innate and acquired components of the immune system [3].

What do PHA results mean?

The PHA test has become a widespread measure of immunocompetence in wild birds, but it must come with an appreciation of what it is able to tell us, and Martin *et al.* 'caution against interpreting larger swellings as 'greater cell-mediated immunocompetence', given the complex nature of the response' [3]. Their findings are important because they reveal a need to distinguish between innate inflammatory reactions resulting from raw tissue damage, and acquired immune responses, when making any inferences about immunocompetence. It could be that an individual produces a swelling proportional to its available body resources, which is useful. However, if the PHA test mainly reflects the innate immune response, then its interpretation will be subject to the same limitations as the previously mentioned monitoring techniques.

PHA-induced swelling is likely to measure, at least in part, a rapid-onset, non-specific inflammatory and innate immune response to tissue damage. The results of Martin *et al.* revealed lymphocytes appearing at the injection site, although B and T lymphocytes were not distinguishable. If there is a contribution to the swelling made by T lymphocytes of the acquired immune response to PHA as a foreign material, as recognised by Martin *et al.*, then it is likely to be at a much lower level on first than on subsequent exposures. Few studies have compared primary and secondary exposures to PHA in the same individual, and, among those that have, the results are inconsistent; some studies found a greater swelling in the secondary than in the primary response, indicative of a true acquired immunity [7,8], but others did not [9]. This inconsistency has arisen even within the same species, depending on what side of the bird was measured [10]. It seems, therefore, an oversimplification to consider the PHA test as an effective and reproducible measure of the acquired, memory immune system, or even the T cell aspect of it.

One important implication of Martin *et al.*'s results is that the PHA response confounds any discrimination between innate and acquired immunity, and future studies will need to disentangle these components. The discrimination between the innate and acquired immune responses is not an idle pursuit. Although the two systems are highly integrated, it is possible that they are separately modified and resourced during different life-cycle stages, particularly at times of high energy demand or enhanced disease transmission. Indeed, it has been shown that selection of chickens for strong innate responses was to the detriment of their acquired immunity [11]. Hence, a strong PHA response might mean a relatively poor acquired immunity.

Methodological perspectives

There has been some discussion about several methodological issues of the PHA test, concerning spatial and temporal repeatability of the measurements [10] and the need for a control injection site [12]. Martin *et al.*'s results showed that little infiltration occurred in saline-injected wings in their study organism. One might therefore risk only a single injection in new studies, although at the expense of a more robust experimental design and controlling for accidental infections, or tissue damage, or operator variability at the experimental injection site.

An additional worry is that the exact PHA response depends on the presence of appropriate cell-surface glycoproteins in the different cells of the injected tissue (Box 1). However, ecological studies usually used preparations that contain a mixture of PHA lectins with different cell specificities, which will bind and potentially stimulate many different cell types (search <http://www.sigma-aldrich.com> for 'PHA'). The exact PHA preparation used in a particular study organism is therefore important and the type of PHA used should always be detailed, and perhaps even standardised between studies. Differences in PHA preparations could explain disparities between studies, and must be considered as a potentially confounding factor in comparative analyses.

If it is important to examine the acquired immune system, then how can this be done? One approach would be to measure directly the antibody response to an injected antigen (such as PHA itself), or the application of contact-sensitising agents that focus preferentially on T lymphocyte and natural killer cell responses [13]. These agents, such as oxazolone or dinitrofluorobenzene, are painted onto small patches of skin. After an appropriate period to enable the T cell response to develop and mature, the agent is applied again, but to a different area of skin, and the response quantified as an increase in skin thickness as for the PHA test. This delayed hypersensitivity response involves neither physical damage to the skin, nor the risk of accidental introduction of infection associated with needle inoculation, and the need for control injection sites.

Conclusion

The sheer complexity and integration of the immune system provides a daunting challenge to field biologists

wishing to understand resource allocation to different physiological systems. However, given the high level of physiological resources requisitioned by the immune system in infection, both for immediate use and for the maintenance of lifelong immunity, it might be timely to rethink what the best tests of immunity in the ecological context might be [14]. The new study by Martin *et al.* should also prompt us to make similar baseline measurements on the effects of PHA exposure in our study organisms to better understand, and place in appropriate context, the immunological responses that we measure.

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