Phytohemagglutinin Skin Test Responses to Evaluate *In Vivo* Cellular Immune Function in Rats (42838)

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Abstract. **It is often necessary to have a small animal model which permits the sequential evaluation of functional immune status over a period of time. We report here the *in vivo*, intradermal response to phytohemagglutinin which produces an area of induration that is histologically similar to a typical delayed cutaneous hypersensitivity response, and that provides fast, quantitative, reproducible results similar to those observed with standard but more laborious and variable *in vitro* tests of immune function. For small animal studies this has the advantage of permitting longitudinal evaluations over time without sacrificing the animal. Using phytohemagglutinin-microprotein (0.2 mg/0.1 ml), injected intradermally, a delayed cutaneous hypersensitivity-like response is induced which is maximal at 24 hr. When immune function was altered either by treatment with a chemical immunosuppressant (ethanol) or by hormonal manipulations (hypophysectomy and rat growth hormone), the delayed cutaneous hypersensitivity-like response (area of induration) correlated closely with both macrophage migration inhibitory factor changes ($r = 0.98$; $P < 0.001$) and mixed lymphocyte reaction changes ($r = 0.99$; $P < 0.05$). These observations suggest that this technique correlates well with standard *in vitro* measures of immune response and may thus permit an *in vivo* estimation of immune reactivity.** [P.S.E.B.M. 1989, Vol 190]

Immune function is known to be extremely sensitive and responsive to a variety of environmental, chemical, and infectious agents, as well as susceptible to changes in nutritional and hormonal factors involved in immune modulation. The *in vivo* evaluation of immune function has been reported using a delayed cutaneous hypersensitivity (DCH)-like intradermal response to phytohemagglutinin (PHA) in human subjects with herpes zoster infection (1), immunosuppression associated with old age (2), and immunosuppression resulting from malnutrition before and after therapy (3). However, an application of this test to an animal model has not been previously described.

This study reports our experiences: (i) in characterizing an *in vivo* rat model in which an intradermal skin test with phytohemagglutinin-microprotein (PHA-M) was employed and (ii) describes the correlation of this *in vivo* PHA-induced skin response with established *in vitro* measurements of immune function, i.e., macrophage migration inhibitory factor (MIF) activity and mixed lymphocyte reactions (MLR).

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Materials and Methods

To establish the optimum intradermal dose for PHA, 12 mature, male Sprague-Dawley rats (obtained from Taconic Laboratory, Germantown, NY) were randomly selected as the experimental subjects. A 5-cm area on the interscapular surface of the back was shaved, depilated, and cleaned. A series of varying doses of PHA-M (Difco Labs, Detroit, MI) ranging from 0.05 to 0.50 mg/0.1 ml of allergy skin test diluent (Center Labs, Port Washington, NY) were injected intradermally. From this was determined the lowest dose capable of producing an area of induration with a diameter >5 mm in more than 90% of the animals at the time of maximum response.

The magnitude of the response following PHA injections was quantified in duplicate (right and left side on the same animal) by measuring the areas of induration resulting from the intradermal injections. Area is expressed in mm^2 calculated as the area of an ellipse (Area = $M/2 \times N/2 \times \pi$, where M and N are

the diameters of the ellipse). *M* and *N* measurements were performed with a small flexible millimeter ruler.

To test the reproducibility of the technique, 15 animals were evaluated in this manner twice at 3-day intervals over a 1-week period. The histologic nature of the reaction was established using full-thickness skin punch biopsies at the time of maximum response in five representative animals.

To document that this *in vivo* PHA skin test response was a valid approximation of immune function, two subsequent experiments were performed in which both DCH-like skin test reactivity to PHA as well as standard *in vitro* tests of cellular immune function were assessed and the observed immune responses compared. To compare the DCH-like responses from PHA to MIF activity following concanavalin A (Con A) stimulation, a group of 14 animals were evaluated in which immune responses were modulated by the acute administration of ethanol (4). Doses of ethanol were varied from 1.0 g/kg to 6.0 g/kg and administered daily for 8 days. In this manner, changes in the PHA-induced DCH-like response could be compared with changes in MIF. MIF activity was measured by standard techniques (5–7). Briefly, spleens were removed from these rats and single-cell suspensions were made by passage through sterile stainless steel screens. The spleen cells at a concentration of 2×10^6 cells/ml were incubated in the presence of serum-free medium alone (RPMI 1640, 1 m*M* L-glutamine, and antibiotics) or medium plus Con A (1 μg/ml) for 3 days. Every 24 hr the supernatants were removed and the cells refed. The pooled supernatants from the 24-, 48-, and 72-hr collections from each individual animal were dialyzed against sterile distilled water for 18 h and lyophilized.

Peritoneal exudate cells were prepared from intact strain 2 guinea pigs previously injected intraperitoneally with 10 ml of sterile mineral oil. The cells were washed and diluted in medium containing 10% guinea pig serum and resuspended at a final concentration of 2×10^8 cells/ml.

Test plates were prepared using plastic petri dishes containing 0.7% Noble agar, 20% normal strain 2 guinea pig serum, antibiotics, 1 m*M* L-glutamine, and $NaHCO_3$ for pH correction. The freeze-dried supernatants were reconstituted in medium at 1/15 of the original volume. Individual supernatants (30 μl) from cells cultured either in medium alone (control) or from Con A-stimulated cells (mitogen) and peritoneal exudate cells (50 μl) were combined. From this mixture 12 μl were dispensed into 3-mm wells in the agar plates and incubated at 37°C in a humid 5% CO_2 incubator for 48 hr. After heat adherence of the cells to the plates at 85°C, the agar was floated off the plate, and the area of the cell migration was measured using a projecting microscope and a compensating planimeter. Percentage of migration inhibition was then calculated as follows:

$$1 = \frac{\text{Area of migration (mitogen)}}{\text{Area of migration (control)}} \times 100$$

In another experiment DCH-like skin test responses were compared with MLR. The immune responses were altered by growth hormone administration in hypophysectomized (HPx) animals. In this manner, changes in DCH-like responses could be compared with changes in MLR.

Twenty-four male Sprague-Dawley rats (body weight 200 g) were maintained on a 13:11 light cycle (lights on for 13 hr and off for 11 hr). Under Brevital anesthesia, the animals were HPx through the right ear canal, or sham HPx, using a Stolting hypophysectomy machine. Postoperatively, animals were allowed to recuperate for 2 weeks before use.

During the recovery and experimental periods, the hypophysectomized rats received 5% dextrose in 0.9% saline to drink. Hypophysectomized animals were then divided into two groups as follows: Group A (HPx) and C (HPx). Group C (HPx) received injections of 50 μg/day of growth hormone in buffered saline for 5 days prior to skin testing. Groups A (HPx) and B (Sham controls) received only buffered saline injections for 5 days prior to skin testing. Skin test responses were measured at 24 and 48 hr after injection of PHA, and animals of all groups were then sacrificed and the thymuses removed (8) and placed in sterile medium. MLR were run as previously described by Sopari *et al.* (9) and Endho *et al.* (10). Stimulator cells were prepared from the spleens of Fisher 344 strain rats (obtained from Harlan Industries). Responder lymphocytes were collected from the thymuses of the Sprague-Dawley rats by washing with sterile medium. Stimulator cells and responder cells were both washed and the stimulator cells were resuspended at a concentration of 2×10^6 cells/ml. Mitomycin C (25 μg/ml of cells) was added to the stimulator cells and incubated for 30 min at 37°C in a 5% CO_2 atmosphere, after which the cells were washed and resuspended again at a concentration of 2×10^6 cells/ml. Responder cells were resuspended at a concentration of 1.5×10^6 cells/ml in medium containing L-glutamine, antibiotics, and 10% castrate adrenalectomized bovine serum. Stimulator cells (0.1 ml) and responder cells (0.1 ml) were pipetted into the wells of round-bottom microtiter plates (A/S Nunc, Roskilde, Denmark) and incubated for 4 days at 37°C in 5% CO_2. On Day 4, 1 μCi ^{3}H-thymidine was added to each well and the incubation continued for an additional 16 hr. The cells were then harvested with a MASH unit and the amount of incorporated radioactivity was determined in a Beckman liquid scintillation counter.

The assays for MIF and MLR required sacrificing the animals; hence, they were performed within 1 hr after reading the skin tests. Analyses were performed in an adjacent laboratory by a research assistant who was

blinded with regard to the skin test results. All data are expressed as the mean ± SEM. For comparisons between groups Student's t test was used. A P value of 0.05 was taken as the upper level of significance.

Results

The initial experiment (Experiment 1) indicated that the size of the area of induration was dependent upon the dose of PHA administered, such that 0.2 mg in 0.1 ml (2 mg/ml) injected intradermally produced a wheal >5 mm in diameter (area ≥ 20 mm^2) in 90% of the animals tested. The peak area of induration was observed at 24 hr (356 ± 85 mm^2) and was progressively diminished in size by 48 hr (282 ± 80 mm^2) and by 72 hr (112 ± 36 mm^2). All subsequent studies (Experiments 2 and 3) utilized this dose and time.

To evaluate reproducibility, animals were studied by repeated testing. From a mean initial area of 83 mm^2 of induration, the mean difference in immune responses in the same animal between testing was −2.27 mm^2 (range, −8.6–9.5). This represents a 2.6% variation between sequential testings. There was no difference between the tests (P = 0.81) when a paired t analysis was performed.

Full-thickness skin punch biopsies at the time of maximum response after intradermal PHA injections confirm the nature of the cellular response. Biopsies reveal a diffuse, predominantly lymphocyte infiltration involving the entire skin thickness and consistent with a delayed cutaneous hypersensitivity (Type 4) response.

Changes in *in vivo* DCH-like skin test response to PHA were then compared with standard *in vitro* tests of immune function. In the first experiment, the variations in host response were induced by daily acute administration of ethanol for 8 days. The correlation between DCH-like skin test response after PHA and MIF activity after Con A stimulation is shown in Figure 1. A highly significant correlation was observed, r = 0.88; $P < 0.001$.

In a third experiment, immune response was altered by changes in the hormonal regulation of the immune system (11–14). This was accomplished using the previously described technique of hypophysectomy and the subsequent readministration of rat growth hormone. The correlation between DCH-like skin test responses to PHA and the mixed lymphocyte reaction is shown in Figure 2. Again a significant correlation was observed, r = 0.93; $P < 0.05$.

Discussion

PHA has long been used as a nonspecific *in vitro* mitogen which stimulates blast transformation primarily of human T lymphocytes (15). In the rat, PHA was also shown (16) to be an *in vitro* lymphocyte mitogen. Its application *in vivo* in man has also appeared in a limited number of reports to evaluate immune status (1–3). To our knowledge, however, its use *in vivo* in animal studies has not been reported previously. Our studies indicate that the area of induration which develops after intradermal PHA is associated histologi-

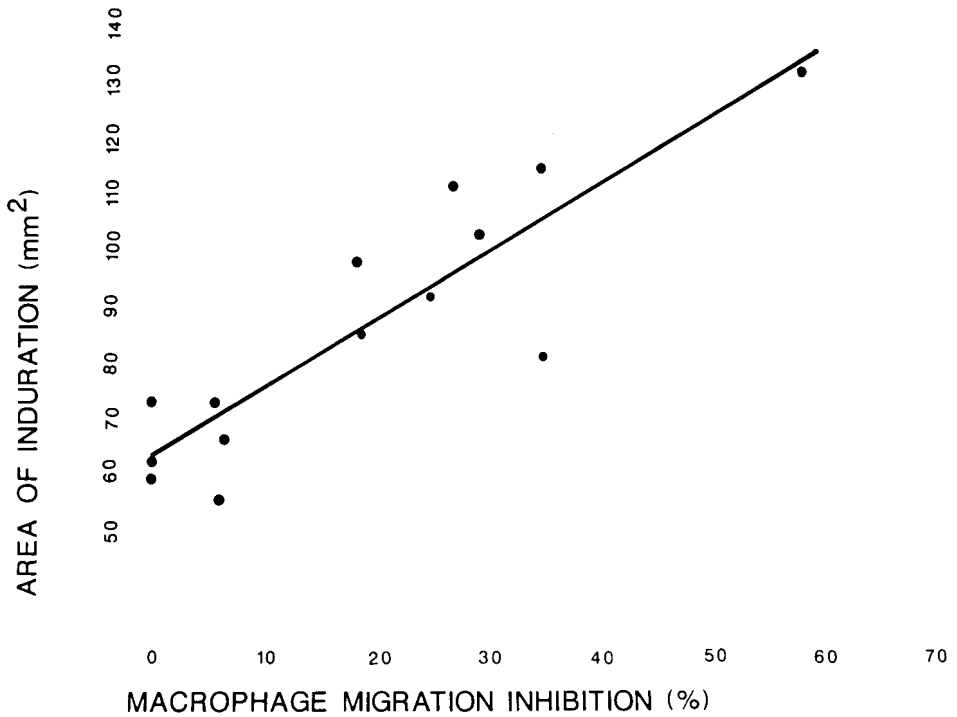

Figure 1. Correlation between the *in vivo* delayed cutaneous hypersensitivity response to an intradermal injection of 0.2 mg of PHA expressed as the elliptical area (mm^2) of induration *vs* the migration inhibition of intact guinea pig peritoneal exudate cells (% inhibition) by supernatant from cultured rat splenic lymphocytes stimulated with Con A (1 μg/ml). n = 14, r = 0.88, $P < 0.001$.

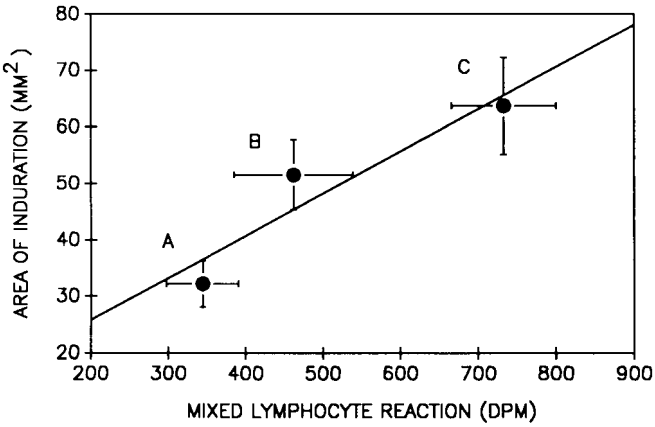

Figure 2. Correlation between the *in vivo* delayed cutaneous hypersensitivity response to an intradermal injection of 0.2 mg of PHA expressed as the elliptical area (mm^2) of induration *vs* ^{3}H-thymidine uptake in a mixed lymphocyte culture in which the stimulator cells were obtained from Fisher 344 rat splenic lymphocytes previously treated with Mitomycin C (0.05 mg/0.1 ml). The responder cells were derived from rat (Sprague-Dawley) thymic lymphocytes from animals that were previously treated as A = hypophysectomy (n = 8); B = sham-operated controls (n = 10); C = hypophysectomy retreated with rat growth hormone (n = 6). Each point represents the mean ± SEM (T) for each of the treatments; $r = 0.93$, $P < 0.05$, $Y = 0.08\ x + 10.52$.

cally with an infiltration of lymphocytes into the area consistent with a DCH-like reaction. Furthermore, the observed *in vivo* response to PHA occurring after either hormonal manipulations or immunosuppression from ethanol produced an area of induration which correlated closely with standard *in vitro* tests of immune function (MIF and MLR).

From these data, we cannot determine the mechanism of the modulation of the PHA skin test response since factors unrelated to immune function may influence skin test responses. Nor is it known whether these responses are etiologically related to observed changes in true DCH reactions which occur after injecting antigen such as purified protein derivative or other moieties requiring presensitization. However, the observed *in vivo* PHA skin test results closely parallel standard *in vitro* tests of cellular immune function, i.e., MIF (a particular *in vitro* correlate of DCH) and MLR (a measure of blast transformation).

These studies exemplify the use of the PHA skin test in a rodent model which provides data that correlate with those changes occuring in the more standard tests of immune function, i.e., MIF and MLR. The test is inexpensive, simple to apply, and rapidly yields quantitative, reproducible results which reflect the cellular immune status of the animal without the need for prior antigenic sensitization, sacrifice of the animal, or blood sampling. This model can easily be applied to the study of immune-modulating drugs, chemicals, infectious agents, or dietary constituents that may alter host response.

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