PHYTOHEMAGGLUTININ (PHA) SKIN TEST.
CHARACTERIZATION OF IMMUNOLOGICAL PROPERTIES
AND CLINICAL APPLICATION

BY

Takaaki Nishido

ABSTRACT

Immunological properties of phytohemagglutinin (PHA) skin reaction were investigated by animal and clinical experiments.

In the guinea pigs an intradermal dose of PHA-P produced erythema and induration with a maximal response at 24 hours after the injection. Histologically it was characterized by perivascular infiltration of lymphoid cells in the dermis and subcutis, being similar to that of tuberculin (PPD) skin reaction. PHA skin reaction, however, showed some difference from that of PPD in the initial cellular response in that the former was composed of small mononuclear cells and granulocytes with rapid development and the latter was composed of large mononuclear cells (macrophages) and granulocytes with slow development.

Intradermal injection of 1:100 dilution of PHA-P produced a similar erythema in man. In 39 of 59 patients with connective tissue diseases, the results of the in vivo (skin test) and in vitro (lymphocyte transformation) response to PHA correlated well. In the 39 patients, the incidence of the positive rate of the PHA tests (55.9%) was significantly higher than that of the DNCB test (33.9%) and of the PPD test (25.7%).

These observations suggest that the PHA skin test has properties of delayed hypersensitivity and is highly sensitive and that it may be a useful measure of cell-mediated immunity.

INTRODUCTION

Delayed hypersensitivity skin reaction has been widely accepted as an in vivo measure for cell-mediated immunity.\(^1\) Purified protein derivative (PPD) of tuberculin, fungus antigens, streptokinase-dornase and dinitrochlorobenzene (DNCB) are the commonly used antigens of the delayed-type skin tests.\(^2,3\) In vitro lymphocyte transformation by phytohemagglutinin (PHA) or other mitogens has also been a common method for examining cell-mediated immunity.\(^4,5\)

In 1963 Schrek and Stefani\(^6\) first reported that an intradermal dose of PHA produced an erythematous lesion similar to the delayed-type skin reaction. Airo\(^7\) described that the histology of the PHA skin reaction, consisting of perivascular infiltration of mononuclear cells, was compatible with that of the delayed-type. The observations by Burgio\(^8\) that infants as well as children responded well to the intradermal PHA with age dependency, suggested the possible availability of the PHA skin test for detecting deficient cell-mediated immunity. There-

---

*1 Some parts of this article were reported at the 18th General Meeting of the Japanese Society of Rheumatism in May, 1974, and at the 2nd General Meeting of the Japanese Society of Clinical Immunology in June, 1974.

*2 西戸孝昭: First Department of Internal Medicine (Chief: Prof. H. Momoi), Faculty of Medicine, Tokyo Medical and Dental University (Tokyo Ika Shika Daigaku).
Received for publication, November, 15, 1978.
after the PHA skin test has been studied mostly in the infant patients with immunodeficiency syndromes, such as Wiscott-Aldrich syndrome,\textsuperscript{9} congenital rubella syndrome,\textsuperscript{10} dysgammaglobulinemia and Nezelof's syndrome,\textsuperscript{11} in which most of the patients showed a negative or weak response. Recently Kataria\textsuperscript{12} observed that most of the patients with sarcoidosis, except for some severely anergic cases, responded well to PHA in vivo. The PHA skin test has also been applied as a trial for the patients under cancer immunotherapy to assess the therapeutic effect on cell-mediated immunity.\textsuperscript{13}

Only a few comparative studies have been made on the PHA skin test with the other in vivo or in vitro parameters of cell-mediated immunity. Blaese\textsuperscript{14} suggested that there is some degree of correlation between the PHA and the other conventional skin tests, and Lowler\textsuperscript{15} also suggested that there is a correlation between the in vivo and in vitro response to PHA.

However, details of the properties of the PHA skin test appear to have not yet been clarified. Therefore, further studies on the precise characterization of the PHA skin test should be required before the routine clinical application.

From such a viewpoint, the present study was designed as follows: (1) In animal experiments the gross and histologic features of the PHA skin reaction were investigated, and (2), clinically, the results of the PHA skin test in the patients with connective tissue diseases were compared with those of in vitro lymphocyte transformation by PHA and of PPD and DNCB skin tests.

**Materials and Methods**

1) Animal experiments

Animals: Male guinea pigs (English Hartley strain) weighing 300 to 500 gm were used.

PHA skin test: PHA-P (Difco) in a vial was dissolved in 5 ml of physiological saline to prepare a stock solution. An intradermal dose of 0.1 ml of 1:100 or 1:500 dilution of the PHA-P stock solution was injected into the back of the guinea pigs, using an equal volume of physiological saline as a control. The skin was observed for erythema and induration and biopsied at appropriate intervals of time as shown in the results.

PPD skin test: The test was performed by the intradermal injection of 0.1 ml of medium strength of PPD (5 μg/ml, BCG, Co.) into the back of the guinea pigs which were pretreated by the injection of 0.1 ml of complete Freund's adjuvant in each posterior or foot pad two weeks previously. The skin lesion was biopsied at the same intervals as the PHA skin test.

DNCB skin test: The skin was challenged by applying 0.1% DNCB in acetone to the back of the guinea pigs which were sensitized by applying 10% DNCB in acetone to the hip two weeks previously. The erythematous area of the skin was biopsied at the same time intervals as the former two tests.

Histological examination: The biopsied materials of each test were fixed by 10% formalin in phosphate buffer and embedded in parafin. The sections (6 μ in thickness) were stained with hematoxylin-eosin for routine examination or by McNemara's modification of Giemsa staining for the analytical examination of the cellular component.

Quantitative analysis of the cellular response of the PHA skin reaction: For the characterization of the initial stage of PHA skin reaction, the intensity of the cellular response and the size of the inflammatory cells every hour for the first six hours were estimated, using a semiautomatic image analysing system (Leitz A.S.M.). For this analytical examination photomicrographs
were taken of five microscopic fields (1:400) from the upper dermis to the dermis-subdermal junction on each preparation and were further magnified when printing to the estimated magnification of 1:3200, which were offered for analysis by Leitz A.S.M.. The results were expressed by the percent ratio of the area of total inflammatory cells to the area of the microscopic field in regard to the intensity of the cellular response and by the mean corpuscular area or the individual corpuscular area in regard to the size of the cells, which were compared with those of the PPD skin reaction.

2) Clinical experiments

Cases examined: Ten normal adults and 59 patients with connective tissue diseases and related diseases, consisting of 20 cases of systemic lupus erythematosus (SLE), 16 of Sjögren’s syndrome (SjS), 11 of rheumatoid arthritis (RA), 5 of progressive systemic sclerosis (PSS), 4 of polymyositis and one each of rheumatic fever, Wegener’s granulomatosis and interstitial pneumonitis with rheumatoid manifestations, were tested.

PHA skin test: According to the method of Lawlor, an intradermal dose of 0.1 ml of a 1:1000 dilution of the PHA-P stock solution was given at the flexor site of the forearm. Erythema and induration were observed sequentially in the normal adults. In the patients the diameter of the erythema at 24 hours was measured and 10 mm or more was considered as positive, according to the results of the normal subjects (details shown in the results).

PPD skin test: According to the method standardized by Seibert, 0.1 ml of the 0.5 μg/ml solution of PPDs (a standard solution, BCG Co.) was given intradermally at the flexor site of the forearm. An erythema with a diameter of 10 mm or more after 48 hours was considered as positive.

DNCB skin test: A modification of the method of Mizoguchi was used. The patients were sensitized by applying a patch containing 100 μg of DNCB to the ulnar site of the upper arm for 24 hours and were challenged two weeks later by applying a patch containing 10 μg of DNCB to the flexor site of the forearm for 24 hours. The appearance of an erythema with vesicles or induration after another 24 hours was considered as positive.

PHA-induced lymphocyte transformation: According to the method of Valentine, peripheral blood lymphocytes were separated from 10 ml of heparinized blood by gravity sedimentation, washed twice with Eagle’s MEM and suspended at the concentration of 5–10 x 10⁶ cells/ml in Eagle’s MEM supplemented with 20% heat-inactivated calf serum. The cell suspension with an addition of 0.05 ml/ml of 1:10 dilution of the PHA-P stock solution was cultured for 72 hours at 37°C in a humidified condition of 5% CO₂ in air. On the smear of the cultured cells by Wright staining, the percent blastoid cell count was made.

Results

1) Gross and microscopic findings of the PHA skin reaction in the guinea pigs: The skin reaction to PHA consisted of erythema and induration. As shown in Fig. 1, the maximal response was attained 24 hours after the injection, when the cutaneous lesion was characterized by an erythematosus area with moderate induration. The cutaneous change following the injection of the 1:500 dilution of PHA was somewhat milder and cleared earlier than that following the injection of the 1:100 dilution.

The PHA skin test was repeated once a week for 4 weeks. Virtually the same result was obtained with each test, revealing that the skin reactivity remained unchanged after repeated injections of PHA.
The lymphocytes in the peripheral blood of the guinea pigs were shown to respond well to the PHA in vitro.

Microscopic findings of the skin after the injection of PHA (1:500) are shown serially in Fig. 2. At 6 hours, a mild infiltration of small round cells and granulocytes was detected in the perivascular area of the dermis and cutis-subcutis junction. The cellular response was intensified, extending to the interstitium at 12 hours and involving the entire dermis and contiguous fatty tissue at 24 hours and then subsiding gradually and clearing fairly well at 48 hours. The established lesion of the PHA skin reaction was characterized by the perivascular infiltration of lymphoid cells and small amount of granulocytes, which was very
similar to that of the PPD skin reaction shown in Fig. 3. The changes in the DNBC skin reaction were principally identical with the former two but the cellular response was more intense in the upper dermis (Fig. 4).

However, an earlier change in the PHA skin reaction appeared to be somewhat different from that of PPD. The PHA skin reaction at 3 hours was composed of small round cells (mostly lymphocytes) and granulocytes, whereas the PPD skin reaction at the same time consisted of large mononuclear cells (probably macrophages) and granulocytes. On the basis of these findings, the following studies on the changes during the initial period of the PHA and PPD skin reaction were performed.

2) Characterization of the initial cellular response of the PHA skin reaction by the semiautomatic image analysing system (Leitz A.S.M.): The size of the lymphocytes, granulocytes and macrophages (large mononuclear cells) was estimated on about 40 selected cells. The frequency of the cells of each size at 1 μ2 intervals is shown in Fig. 5, where the mean size of the lymphocytes was 4.8±2.1 μ2, granulocytes 8.3±2.7 μ2, and macrophages 11.0±2.9 μ2.

Fig. 6 shows the serial changes in the intensity of the cellular response of the PHA and PPD skin reaction as indicated by the percent ratio of the summed area of the inflammatory cells to the area of the microscopic field. The cellular response develops more rapidly with PHA than PPD. The serial changes in the mean size of the inflammatory cells in the PHA and PPD reaction are shown in Fig. 7, where the mean size of the cells in the PPD reaction was greater in four of six occasions (1, 3, 4 and
Fig. 5. Size-distribution of lymphocytes, granulocytes and macrophages analysed by Leitz A.S.M., each for about 40 cells selected randomly from the photomicrographs.

Fig. 6. Development of the cellular response in the initial stage of PHA and PPD skin reaction. Intensity of the cellular response is expressed as the percent ratio of the summed area of the inflammatory cells to the area of a field of microscopic view, estimated by Leitz A.S.M.
6 hours) than that in the PHA reaction. This may indicate the difference of the cell types participating in the early response of these skin reactions. Then the size-distribution of the mononuclear cells and polymorphonuclear cells (granulocytes) in the PHA and PPD skin reaction at 3 and 6 hours was examined (Fig. 8). The difference in the mononuclear cells of both reactions was obvious. The mononuclear cells in the PHA were small in size with a mean value of 4.6±2.2 μ² at 3 hours and 5.1±2.3 μ² at 6 hours, suggesting that these cells were mainly composed of small lymphocytes as shown in Fig. 5. On the contrary, the mononuclear cells in the PPD were larger in mean size (6.7±2.8 μ² at 3 hours and 6.7±3.1 μ² at 6 hours), including the various sizes of the cells composed of macrophages, large lymphocytes and some small lymphocytes.

3) Gross and microscopic findings of the PHA skin reaction in man: The time course of the PHA skin reaction in man was essentially identical with that in the guinea pigs. The maximal reaction was attained 18 to 24 hours after the injection (Fig. 9), and the erythema almost faded within 48 hours. Biopsy of the skin at 24 hours revealed a perivascular infiltration of lymphoid cells, especially in the lower dermis and the contiguous fatty tissue (Fig. 10). These histological pictures were almost indistinguishable from those of the DNCB skin reaction (Fig. 11).

In 10 normal adults the mean diameter of the erythema was 11.6 mm, ranging from 10.5 to 14.0 mm. Accordingly the PHA skin test in man was considered as positive when the diameter of the erythema determined after 24 hours was 10 mm or greater. The normal mean value of percent blastoid cells of the lymphocytes in response to PHA was 51.4±9.8% (mean±2 SD).

4) The relation between the in vivo and
Fig. 8. Size-distribution of the inflammatory cells as divided into groups of mononuclear cells (MNC) and polymorphonuclear cells (PMN) in PHA and PPD skin reaction at 3 and 6 hours. All the cells discernible in the photographs were analyzed by Leitz A.S.M.. The numerical values in each column indicate the mean corpuscular area (mean ± 2SD).

Fig. 9. Gross picture of PHA skin reaction in man (at 24 hours).
in vitro response to PHA in man: The in vitro response of the lymphocytes to PHA as indicated by the percent blastoid cells was compared between the patients with positive and negative results of each skin test (Table 1). The mean value of the percent blastoid cells was 40.6±14.8% (mean±2 SD) in 24 cases with a positive result and 30.6±11.4% in 15 cases with a negative result with PHA, 40.8±15.9% in 19 cases with a positive result and 33.0±13.7% in 20 cases with a negative

<table>
<thead>
<tr>
<th>Skin test</th>
<th>No. of cases</th>
<th>Blastoid cells (%)</th>
<th>t-Test</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA</td>
<td>Positive 24</td>
<td>40.6±14.8</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>Negative 15</td>
<td>30.6±11.4</td>
<td></td>
</tr>
<tr>
<td>DNBC</td>
<td>Positive 19</td>
<td>40.8±15.9</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>Negative 20</td>
<td>33.0±13.7</td>
<td></td>
</tr>
<tr>
<td>PPD</td>
<td>Positive 10</td>
<td>39.6±12.4</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>Negative 29</td>
<td>35.8±17.6</td>
<td></td>
</tr>
</tbody>
</table>
result with DNCB and 39.6±12.4% in 10 cases with a positive result and 35.8±17.6% in 29 cases with a negative result with PPD. The difference was highly significant (p<0.001) for the PHA test and also significant (p<0.01) for the DNCB test, but not significant for the PPD test. A good correlation between the in vivo and in vitro response to PHA was evident.

5) Incidence of the positive skin test with PHA, DNCB and PPD (Table 2): In 59 cases with collagen diseases, the skin test was positive in 55.9% with PHA, being significantly higher as compared with 33.9% with DNCB and 27.3% with PPD. On the contrary, of the 33 patients with non-collagen diseases, the PHA test was positive in 69.7%, 66.7% positive with DNCB and 51.5% positive with PPD, there being no significant differences.

In 6 of the 20 cases with SLE the test was positive with PHA and only one was positive with DNCB, but none responded to PPD. One of the 6 was in the early stage and the others were in the remission stage. In the patients with diseases other than SLE, most cases responded to PHA but less than a half responded to DNCB and PPD. The results indicate the high sensitivity of the PHA skin test.

6) Relation among the three skin tests (Table 3): The positive incidence of DNCB and PPD skin test was examined in the patients with positive PHA skin tests. In 33 patients with collagen diseases showing a positive PHA test, 16 cases (48.5%) were positive with DNCB and 12 (36.4%) was positive with PPD, while in the non-collagen diseases 18 (78.3%) of 23 cases with a positive PHA test responded to DNCB and 13 (56.5%) to PPD.

7) Changes in skin reactivity during the course of illness: The skin tests were repeatedly examined in some patients. Illustrative cases are presented below.

Case 1. A 47-year-old woman suffering from SjS for ten years had showed varieties of immunological abnormalities including negative response to all the skin tests on the first admission in May 1973. On the second admission (September 1973) because of the development of reticulum cell sarcoma, the skin tests still remained negative and six months after anti-neoplastic therapy the PHA and DNCB tests became positive again. In September 1974, when lymphadenopathy recurred with minor abnormalities in the immunological findings, only the PHA skin test was positive. The PPD skin test remained negative until the complication of pulmonary tuberculosis occurred, when the PHA test was again positive but the DNCB test was negative.
Case 2. A 22-year-old woman with SLE was readmitted in May 1974 because of the relapse of lupus nephritis and psychosis, when a positive LE cell test and marked hypocomplementemia were found and all the skin tests were negative. The PHA and DNBC tests became positive again one year after the steroid therapy, while the PPD test remained negative throughout the course of the disease.

Discussion

The biological and chemical properties of PHA had been reviewed precisely by Naspiiz and Richter, who also indicated that an intradermal dose of PHA causes an erythematous skin reaction similar to that of delayed hypersensitivity. Schrek and Stefanii first described that an intradermal injection of 0.001 ml of PHA solution induced an erythema and induration in man and guinea pig 24 hours after injection and that the in vitro and in vivo response to PHA may be a delayed hypersensitivity reaction.

The present study was designed to characterize precisely the PHA skin reaction and to study the clinical significance of the test in the collagen diseases, an important group of immunological diseases, on which the PHA skin test has never been tried.

Our experiments showed that the PHA skin reaction in man and guinea pig showed a gradual development of erythema and induration with a maximal response about 24 hours after the injection and that the histological changes of the maximal reaction were principally composed of perivascular accumulation of lymphoid cells, which is compatible with the characteristics of delayed hypersensitivity skin reaction represented by the tuberculin skin reaction.

In addition, noticeable observations were obtained newly in that the manner of the initial response of the PHA and PPD skin reaction appears to be somewhat different from each other. An analytical investigation of the cellular response during the initial 6 hours of the PHA and PPD skin reaction by a semiautomatic image analysing system (Leitz A.S.M.) revealed that the cellular response to PHA as indicated by the percent area of the total inflammatory cells develops more rapidly than to PPD (Fig. 6) and that the mean size of the cells in the PHA reaction is smaller than that in the PPD reaction (Fig. 7). This difference in the size of the cells may be attributable to the fact that the mononuclear cells in the PHA reaction are composed mainly of small lymphocytes while those in the PPD reaction are composed of macrophages, large lymphocytes and a few small lymphocytes, as was shown by the size-distribution study of these cells (Fig. 8).

These differences between the PHA and PPD skin reaction may be ascribed to the biological nature of each agent, PHA being a nonspecific mitogen and PPD a specific antigen. The action of the PHA on the T lymphocytes requires the participation of the accessory cells, such as macrophages and fibroblasts, but this accessory cell-dependency is not so strict that the PHA may be capable of acting directly on the T lymphocytes possibly to release the chemical mediators (lymphokines) which may play an important role in the development of the cellular response, while the action of PPD is stringently dependent on the macrophages which participate in the antigen recognition and activation of the immune-specific T lymphocytes.

Accordingly it is evident that the distinctive features of the PHA skin reaction are present in the initial stage, though the peak reaction is indistinguishable from that of delayed hypersensitivity.
The evidence for the delayed-type properties of the PHA skin test was the good correlation between the in vivo and in vitro response (Table 1). Lawlor and Stiehm also observed that 12 of the 13 cases with a normal response to PHA in vitro responded well to PHA in vivo, while 6 patients with an impaired response to PHA in vitro showed a negative or weak response to PHA in vivo. In the present study the DNCB skin test also correlated with the in vitro response to PHA, but the PPD skin test did not, which may be attributed to the negative result of the PPD skin test in most of the patients examined.

On the other hand, as described by Blaese, inconsistency in the in vivo and in vitro response to PHA was observed rather frequently in the individual cases of the present series, where most of the patients showed a slight or moderate decrease in the in vitro response to PHA. Airo observed a good response to PHA in vivo in spite of the marked decrease in the in vitro response to PHA in the patients with chronic lymphocytic leukemia and speculated on this contradiction that only a small amount of the lymphocytes with functional integrity may be necessary to cause the skin reaction. The present observation appears to support this notion and further more indicates that the PHA skin test is a sensitive measure of delayed hypersensitivity reaction.

In the comparative study on the skin response to PHA, DNCB and PPD, a positive PHA test was found in over half of the patients with collagen diseases but the DNCB or PPD test is positive in far fewer cases, while in the control group of patients a positive PHA or DNCB test was found in about 70% and positive PPD test in about 50%. However the positive or negative results of the PHA skin test were consistent with those of the DNCB or PPD skin test in over 60% of the cases in both groups. In this respect, Blaese reported on the presence of a good correlation between the PHA skin test and other routine skin tests in the infants and children but not so much in the adults as regards the immunodeficiency syndromes, which may not be incompatible with the present observation. As additional findings shown in the case reports, the parallelism between the PHA skin test and the clinical and immunological features may be a great advantage of this test.

In conclusion, distinctive properties of the PHA skin reaction are present in the initial response and the entire pictures are quite compatible with the delayed hypersensitivity reaction. High sensitivity, correlation with the in vitro response to PHA and a parallelism with the clinical and immunological course are considered to be the clinical characteristics of this test. Practical simple technique and no requirement of prior sensitization may be the great advantages. The PHA skin test may be a useful measure for detecting impaired cell-mediated immunity.

Acknowledgement

The author wishes to thank Mr. K. Sugiyama and Mr. I. Furutani of Sieber Kikai Company for granting permission to use the Leitz A.S.M. and for their technical assistance.

References


**ABBREVIATIONS**

| PHA | phytohemagglutinin |
| PHA-P | phytohemagglutinin P |
| PPD | purified protein derivative of tuberculin |
| DNBC | dinitrochlorobenzene |
| SLE | systemic lupus erythematosus |
| RA | rheumatoid arthritis |
| SJS | Sjogren's syndrome |
| PSS | progressive systemic sclerosis |
| PMN | polymorphonuclear cells |
| MNC | mononuclear cells |