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A New Micromethod for Evaluating Lymphocyte Responses to Phytohemagglutinin: Quantitative Analysis of the Function of Thymus-Dependent Cells

(immunodeficiency/tritiated thymidine)

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ABSTRACT **We have developed a new micromethod for testing phytohemagglutinin stimulation of human lymphocytes. 50 μl of peripheral blood is incubated at 37°C directly, without cell separation, with 50 μl of tissue culture medium containing 50 μg of phytohemagglutinin for 24 hr. Then, 0.5 μCi of tritiated thymidine in 50 μl of tissue culture medium is added and incubated for an additional 16 hr. After incubation, erythrocytes are selectively lysed in distilled water, and white blood cells are trapped quickly on a Millipore filter. The radioactivity of the filter is then assessed by a scintillation counter. The requirement of extremely small amounts of blood and a short incubation time are advantages. Further, this method provides a most useful evaluation of thymus-dependent cell populations in man and experimental animals and, for the first time, this method provides the possibility of quantitating the deficiency of thymus-dependent cells.**

Stimulation of human peripheral lymphocytes by phytohemagglutinin (PHA) has been recommended as a means of testing the over-all function of thymus-dependent cells ("T"-cells) (1).

Conventional methods of executing this test require isolation of lymphocytes from a large volume of blood and a prolonged period (3–7 days) of incubation at 37°C. Thus, it is often difficult to repeat this test frequently, especially in small infants, for precise and prompt evaluation of "T"-cell function. We have developed a new method for evaluation of "T"-cell function by use of PHA that requires very small amounts of blood and about 40 hr of incubation. We believe that this new method may provide a means of quantitating the number of "T"-cells in the circulation.

MATERIALS AND METHODS

0.5 ml of venous blood or capillary blood was collected into a sterile, capped polystyrene tube (12 × 75 mm, Falcon plastics, Oxnard, Calif.), and 5 units of heparin (containing 0.9% benzyl alcohol as a preservative) were added. The blood was mixed thoroughly by gentle tapping of the tube. 50 μl of this blood was added into the sterile, capped polystyrene tube and mixed with 50 μl of tissue culture medium (RPMI 1640, Associated Biomedics, Inc., Buffalo, N.Y.) containing 50 μg of PHA-M (Bacto phytohemagglutinin-M, Difco Laboratories, Detroit, Mich.). To the control tube, 50 μl of blood and 50 μl of tissue culture medium (RPMI-1640) without PHA-M were added. Both PHA and control tests were performed in triplicate. The tubes were gassed briefly with 10% CO_2 in sterile air, capped loosely, and incubated at 37°C with 7.5% CO_2 and humidity for 24 hr, or for appropriate periods as indicated. At the end of this incubation, 0.5 μCi of [methyl-^{3}H]dT (specific activity 2.0 Ci/mol, New England Nuclear, Boston, Mass.) in 50 μl of tissue culture medium (RPMI-1640) was added to each tube and mixed gently by tapping the tube. The tubes were incubated for another 14 hr, or for appropriate periods as indicated, under the same conditions. At the end of the second incubation ([^{3}H]dT pulse), 3 ml of distilled water was added to the culture tube, which was mixed vigorously with a pasteur pipette to induce rapid lysis of the erythrocytes.

The entire mixture was then quickly filtered through a Millipore filter (1.2-μm pore size, 13 mm in diameter, Millipore Corp., Boston, Mass.) by use of the apparatus described by Robbins *et al.* (2), with a minor modification; A side vent was created by passage of a pasteur pipette through a rubber stopper to monitor the reduced pressure inside the erlenmeyer flask. Further, a longer glass specimen vial was used to eliminate the necessity of cementing the screw caps. The filter with entrapped white blood cells and erythrocyte ghosts was washed successively by filtration of 4 ml of cold physiologic saline and 4 ml of 5% trichloroacetic acid. Additional suction was applied for 10–15 sec to remove excess water from the filter paper, and the reduced pressure was released by opening the side vent. The filter paper was carefully transferred with forceps to the bottom of a glass specimen vial (15 × 45 mm, Kimble opticlear, produced by Owens-Illinois Co., Toledo, Ohio); 0.1 ml of 0.2 N KOH was added to dissolve the collected precipitate from the filter paper. The tube was placed in a standard scintillation vial. 30 min later, 3 ml of scintillation solution (see below) was added to the specimen vial; after 2 hr, the radioactivity of tritium was measured in a liquid scintillation counter, Beckman model LS 250. The scintillation solution consisted of 8 g of butyl-PBD (Research Products International Corp., Elk Grove Village, Ill.), 150 g of naphthalene (Eastman Kodak Co., Rochester, N.Y.), 35 g of CAB-O-SIL (grade M5, Cabot Corp., Boston, Mass.), and 1 liter of *p*-dioxane (Matheson, Coleman, and Bell Co., Norwood, Ohio).

The response of lymphocytes to the stimulation by PHA-M was measured by the uptake of [^{3}H]dT into the DNA of lymphocytes. Incorporation of [^{3}H]dT was expressed as cpm per 50 μl of blood. These results were compared for the separate groups. The total white blood cell and differential count were performed by standard methods (14).

Abbreviations: PHA, phytohemagglutinin; T-cells, thymus-dependent cells.

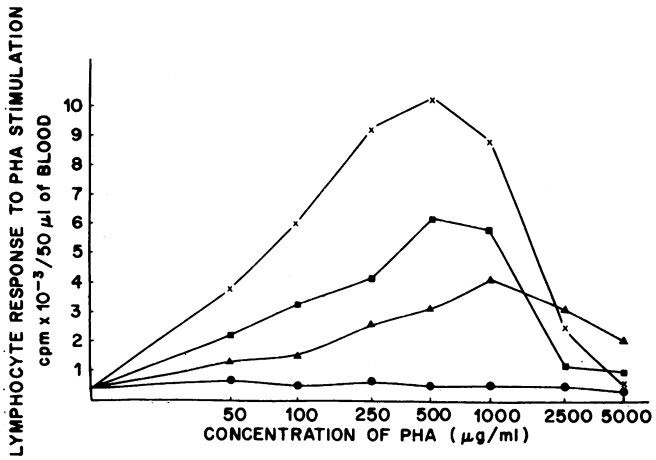

FIG. 1. Lymphocyte response to stimulation by PHA (dose–response curve) 24 hr of incubation at 37°C, 16 hr of [^{3}H]dT pulse. Each point on the curve represents the average of the experiments (in parentheses). ×——×, normal (15 tests); ■——■, common variable form of immunodeficiency (8); ▲——▲, ataxia telangiectasia (3); ●——●, combined immunodeficiency (5).

RESULTS

Incorporation of [^{3}H]dT by lymphocytes in response to the stimulation of PHA-M at various concentration is shown in Fig. 1. The maximum response was noted at a concentration of 500 µg of PHA/ml in normal controls. Lesser response was noted in lymphocytes from patients with ataxia telangiectasia and the common variable form of immunodeficiency. No response was obtained at any time in blood of children with severe combined immunodeficiency of the autosomal recessive type. In the blood from patients with ataxia telangiectasia, the maximum response occurred at a higher concentration of PHA than was the case with blood from normal individuals or patients with common variable form of immunodeficiency. Incorporation of [^{3}H]dT by the lymphocytes of blood at a PHA-M concentration of 500 µg/ml and various periods of incubation is shown in Fig. 2*A*. A plateau was reached after 24–30 hr of incubation when the period of "[^{3}H]dT pulse" was 12 hr.

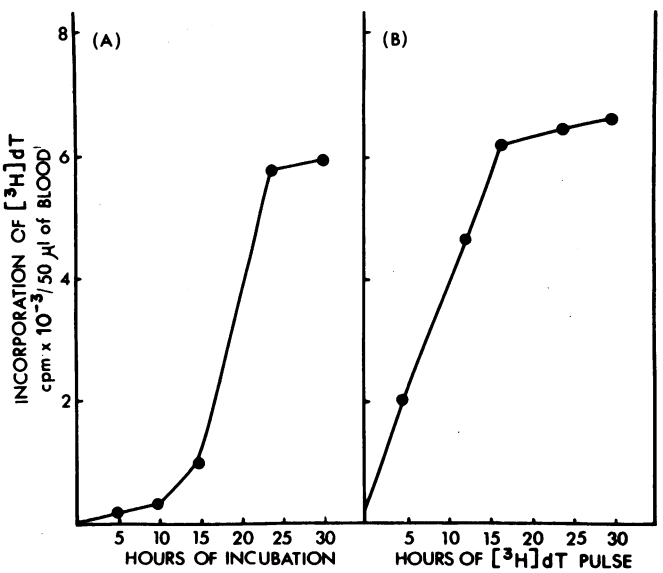

FIG. 2. (*A*) The incorporation of [^{3}H]dT at various periods of incubation at 37°C. 12 hr of [^{3}H]dT pulse (average of 5 Expts.). (*B*) The incorporation of [^{3}H]dT with various durations of pulse. 24 hr of incubation (average of 4 Expts.).

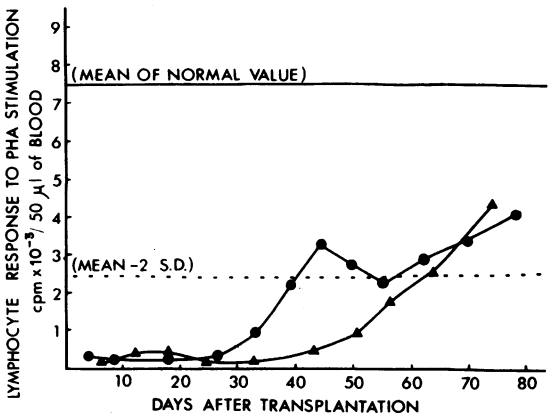

FIG. 3. Restoration of the capacity to respond to stimulation by PHA in patients with combined immunodeficiency disease after bone marrow transplantation. ●——●, 9-month-old female, received fathers bone marrow. ▲——▲, 3-month-old boy, received sister's bone marrow (HLA-identical).

Incorporation of [^{3}H]dT at the PHA-M concentration of 500 µg/ml, incubation period of 24 hr, and various periods of "[^{3}H]dT pulse" is shown in Fig. 2*B*. A plateau was reached 16–20 hr after the start of the "pulse" period.

The results of PHA stimulation in various patients at a PHA concentration of 500 µg/ml, 24-hr incubation, and 16-hr "pulse" is shown in Table 1. A markedly decreased response to PHA stimulation was noted in patients with combined immunodeficiency disease. Patients with agammaglobulinemia of common variable form showed a lesser response than did controls, although in some instances responses were within the normal range. Patients with ataxia telangiectasia showed a variable response but, on the average, tended to have deficient responses.

No direct correlation was found between the vigor of response obtained and the absolute number of lymphocytes in each sample. Immunologic reconstitution of two infants with combined immune deficiency disease by bone marrow transplantation resulted in a gradual increase in the response to PHA stimulation (Fig. 3).

TABLE 1. *A micromethod of PHA-test on human blood*

Group	Age	No. of tests	Responses	
			Control	With PHA
Normal	1 month(s)–52 year(s)	145 (120)*	204 ± 82†	7650 ± 2615†
CID	3 month(s)–13 month(s)	15 (3)	65–520 (215)	82–485 (280)
CVID	6 year(s)–21 year(s)	12 (5)	95–450 (240)	1650–9500 (4850)
A.T.	8 month(s)–9 year(s)	14 (4)	190–600 (310)	1900–8500 (3200)

CID, Combined from of immunodeficiency disease; CVID, common variable form of immunodeficiency disease; A.T., Ataxia telangiectasia; * No. of patients studied (in parentheses); † mean ±1 SD (in parentheses).

DISCUSSION

Lymphocytes in peripheral blood comprise at least five broad functionally different groups of cells; (*a*) thymus-derived cells ("T"-cells), (*b*) bursa-derived cells ("B"-cells), (*c*) monocytes, (*d*) primitive stem cells, and (*e*) "partially" differentiated stem cells (3, 4). At present, it is difficult, if not impossible, to distinguish or separate these subgroups of cells by either histochemical or physical methods. The methods used currently to isolate lymphocytes from peripheral blood inevitably result in random selection of these subgroups, and provide uncontrolled variables in each experiment. Furthermore, the absolute number of lymphocytes in the peripheral blood varies in different persons, and in different days in the same person. Therefore, the current methods of stimulation of isolated lymphocytes by PHA do not provide a quantitative and reproducible means of assessing "T"-cell function. Further, the usual methods involving not only isolation of, but concentration of, the lymphocytes to suitable numbers for assay introduce further uncontrollable variables. Thus, one could have "T"-cell lymphopenia and still show an absolutely normal PHA response by conventional methods. Theoretically, the assay described herein would not only detect but quantitate the degree of "T"-cell deficiency.

In the method described here, whole blood was used instead of isolated lymphocytes, and the degree of response to PHA-M stimulation was expressed in terms of unit volume of blood. Since the steps of the procedure for isolation of lymphocytes are no longer necessary, the possibility of random selection of the subgroups of lymphocytes are eliminated. The blood volume of a given person is constant, and varies little from one person to the other in relation to the body weight. Therefore, the results of day to day experiments and those with blood of different persons can be more comparable.

The presence of erythrocytes did not seem to affect the incorporation of [^{3}H]dT under the conditions of this assay. This is in accord with the findings of others (5). The presence of polymorphonuclear leukocytes has been reported to enhance the transformation of lymphocytes by PHA (6), but to have variable effect on the mixed leukocyte culture reaction (7, 13). Thymidine is catabolized by polymorphonuclear leukocytes (8). We did not find any major effect of the number of polymorphonuclear leukocytes on [^{3}H]dT incorporation. Heparin has also been reported to depress the mixed leukocyte culture reaction, but not the PHA response (9). The concentration of heparin used here did not seem to have any adverse effect, since the results were comparable when the concentration of heparin was varied from 5–30 units/ml.

The majority of peripheral lymphocytes of normal persons are in G_1 phase of the cell cycle (10). The average duration of G_1, S, and G_2 phases of the cell cycle of human leukocytes have been reported to be 4.6, 9.6, and 3.5 hr, respectively; the average generation time for the dividing cells is 17.7 hr (11). Since the incorporation of [^{3}H]dT began at about 12 hr after the commencement of the first incubation, and reached its plateau at 36–40 hr, it is most likely that the first round of the cell cycle is mainly responsible for the incorporation of [^{3}H]dT. Therefore, the amount of incorporated [^{3}H]dT would be directly proportional to the number of "T"-cells responding to PHA-M stimulation in a unit volume of blood.

The dose–response curve for testing "T" cells by stimulation by PHA is similar to that obtained by others (12). The concentration of 500 μg of PHA-M/ml, 24 hr of incubation, and 16 hr of "[^{3}H]dT pulse" represent optimal conditions for the uptake of [^{3}H]dT, and discriminate sharply the normal from abnormal response.

Further studies on the influence of aging, chemical immunosuppressive therapy, "blocking antibodies", antilymphocyte globulin, and other serum factors on the stimulation of "T" cells by PHA must be performed.

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