Viral-Induced Unresponsiveness of Tuberculin-Sensitized Guinea Pig Lymphocytes[1] (35373)

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Peripheral blood lymphocytes cultured *in vitro* with phytohemagglutinin (PHA) or with antigens with which the donor has had prior contact, undergo enhanced macromolecular synthesis and transformation to large blastoid cells (1–3). In general, cultures of peripheral lymphocytes from healthy human beings stimulated in this manner produce a high degree of cellular transformation. However, lymphocytes from patients with diseases such as Hodgkin's disease, chronic lymphatic leukemia, sarcoidosis, agammaglobulinemia and thymic dysplasia show varying degrees of unresponsiveness to *in vitro* stimulation (4–6). These patients also possess decreased cellular immunity; consequently, it has been suggested that blastogenic transformation of the small lymphocyte following stimulation with mitogens and antigens is an expression of cellular immunological competency (4).

Addition of chemotherapeutic drugs (7) and microorganisms such as certain mycoplasma (8) and viruses (9, 10) will impair mitogen- and antigen-induced transformation of competent peripheral lymphocytes. For example, Smithwick and Berkovich (10) showed that measles viruses inhibit the *in vitro* tuberculin-induced transformation of peripheral lymphocytes from Mantoux positive children. Olson *et al.* (9) have demonstrated that rubella virus and Newcastle disease virus (DNV) infections of peripheral lymphocytes from healthy human beings will impair the ability of these cells to respond to *in vitro* stimulation with PHA, pokeweed mitogen, diphtheria toxoid, and tetanus toxoid. The authors suggested that some viruses are capable of invading and producing an unresponsive state in lymphocytes, and that these virus infections could be the etiological cause for depressed immunological function in many diseases of unknown etiology.

The present work done in tuberculin-sensitized guinea pigs confirms the observations that virus infections of sensitized lymphocytes from healthy donors will produce a state of specific antigen-induced unresponsiveness. Moreover, it establishes the guinea pig as an animal model for investigating the virus–lymphocyte relationship. The data show, however, that care must be exercised in evaluating such reactions, because at least in some situations, a natural temporary antigen-induced unresponsiveness may develop following skin testing.

Materials and Methods. Outbred male and female guinea pigs of the Rockefeller strain, propagated in the Department of Microbiology and weighing 700 to 1000 g were used in all experiments. Animals were housed in separate cages and were fed Purina guinea pig chow and tap water supplemented with 0.3% ascorbic acid, *ad libitum*.

Guinea pigs were sensitized to tuberculin by sc injections in the nape of the neck with heat-killed (100° for 1 hr) *Mycobacterium tuberculosis*, $H_{37}RV$ strain, suspended in paraffin oil mixed with melted Vaseline (11). Each animal received 0.75 mg of the heat-killed tubercle bacilli in a total inoculum of 1 ml, injected into 5 sites. Animals were skin tested 4 weeks later, by injecting into the flanks 0.1 ml containing 1 μg of tuberculin, purified protein derivative (PPD, Parke, Davis and Co., Detroit, Lot. No. 98428A) or 1:2500 dilution of old tuberculin (OT, Eli

[1] This investigation was supported in part by funds from a University of Arizona Institutional Grant.

Lilly Co., Indianapolis, Ind.). The diameter of the indurated–erythematous reaction was read and scored after 24 hr according to the procedure described by Chase (11).

At intervals, control and tuberculin-sensitized animals were bled by cardiac puncture without anesthesia. Peripheral leukocytes were separated from the heparinized blood samples (10 units/ml of blood) by the method of Hullinger and Blazkvec (12). Leukocytes were washed twice with Earle's balanced salt solution (Earle's BSS) containing 30 units of heparin, 100 units of penicillin, and 100 μg of streptomycin/ml; and 2.2 g of sodium bicarbonate/liter. After the final wash, the leukocytes were suspended in 2 ml of Earle's BSS and cell concentration and viability were determined.

Cell concentration was adjusted to 8×10^5 viable cells/ml in McCoy's 5A medium (Grand Island Biological Co.) containing 100 units of penicillin, 100 μg of streptomycin, and 30 units of heparin/ml; and 20% fetal calf serum. Cell cultures containing 3 ml of cell-medium suspension were prepared in glass disposable culture tubes with Morton closures. Appropriate cell cultures received reconstituted PHA or various dilutions of PPD and/or NDV preparations in 0.1-ml volumes. Cultures stimulated with PHA and PPD were incubated for 4 days and from 4 to 7 days, respectively, in a 5% CO_2–air humidified atmosphere at 37°.

NDV (Roakin strain Cat. No. V-326-001-000, Chas. Pfizer & Co. Inc.) was used in concentrations of 128 to 0.128 hemagglutination units (HA)/0.1 ml/culture. Stock NDV was prepared according to the method of Henle and Hilleman (13) and purified by the method of Wheelock and Tamm (14). Portions of the purified virus were inactivated with ultraviolet irradation as described by Olson *et al.* (9).

After incubation, cultures were centrifuged at 150*g* for 5 min. Sedimented leukocytes were resuspended, washed twice with Earle's BSS, and then suspended in 0.2 ml of Earle's BSS. Cell viability was determined by the trypan blue dye exclusion method (15). Samples of the dispersed cell preparations were prepared in duplicate on microscope slides and stained with Giemsa stain.

The effectiveness of PHA and specific antigen-induced stimulation and virus inhibition was ascertained by determining the number of blastoid cells in the treated cultures as compared to the control cultures. One thousand mononuclear cells from each of the duplicate slides were counted. The response was recorded as the number of transformed blastoid cells, expressed as percentage, per 1000 mononuclear cell population. The degree of transformation in stimulated and in stimulated virus-infected cultures was determined with the following formulas:

$$\% \text{ increase } (+) = \left[\frac{(\text{Transformation for PPD cultures}) - (\text{Transformation for cultures with no additives})}{(\text{Transformation for cultures with no additives})} \right] \times 100.$$

$$\% \text{ decrease } (-) = \left[\frac{(\text{Transformation for PPD cultures}) - (\text{Transformation for PPD + NDV cultures})}{(\text{Transformation for PPD cultures}) - (\text{Transformation for cultures with no additives})} \right] \times 100.$$

Results. The experimental conditions required for maximum *in vitro* PPD-induced transformation of peripheral lymphocytes obtained from mycobacterium sensitized guinea pigs were determined. Cells were collected from six animals with 4+ skin reactions, 48 hr after skin testing with PPD. These cells were cultured for 5, 6, or 7 days in the presence of 0, 0.05, 0.5, or 5.0 μg of PPD. Results, depicted in Fig. 1, indicate that maximum transformation was obtained after 6 days of culture in the presence of 0.5 μg of PPD. Net cellular transformation was 8%. Consequently, 0.5 μg of PPD/culture, (2.4×10^6 peripheral leukocytes) and an incubation period of 6 days were selected as optimal conditions for subsequent experiments.

Another experimental condition to be ascertained was the effects mycobacterial sensitization and subsequent skin testing with PPD would have upon the ability of lymphocytes to respond to PPD and PHA stimulation *in vitro*. Peripheral cells were collected before and at various times after the guinea pigs were sensitized with heat-killed mycobacterium and skin tested with PPD. All cell

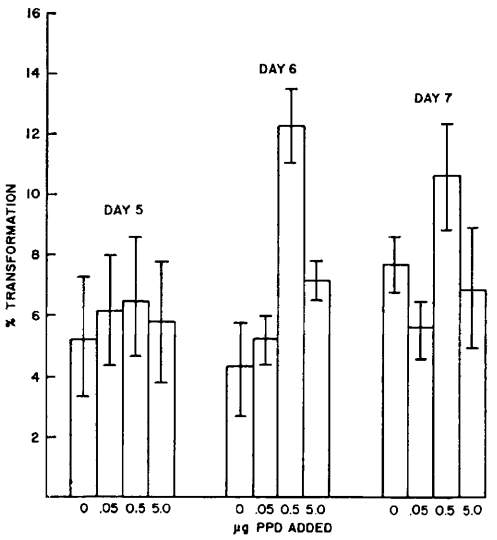

FIG. 1. Dose–response curve for PPD-induced transformation of lymphocytes from guinea pigs sensitized with *M. tuberculosis*.

samples were assayed immediately after collection to determine the number of blastoid cells which if present would indicate specific antigen-induced transformation *in vivo*. Cell cultures were maintained for the optimal time periods of 4 days in the presence of 0.1 ml of reconstituted PHA or for 6 days in the presence of 0.5 μg of PPD.

Figure 2 presents the variations in responsiveness of these cells to specific and nonspecific stimulants as a function of time during sensitization. Differential counts of peripheral leukocytes at zero times show a slight increase in blastoid cells (0 to 1.8%) approximately 1 month after sensitization with mycobacterium. This value returned to zero levels by 40 days and was not influcenced by skin testing. PHA-induced transformation of lymphocytes from four guinea pigs before sensitization was 47%. Two and 14 days after mycobacterial sensitization, the peripheral lymphocytes failed to respond to PHA stimulation *in vitro* as evidenced by decreased transformaiton values of 24.9% and 8.9%, respectively. By day 29, the cells had regained their normal responsiveness to PHA stimulation. A skin test with PPD on day 29 caused a significant decrease in lymphocyte responsiveness to PHA as indicated by the 2.8% transformation of cells obtained on the sixth day after skin testing (35th day postsensitization). Normal PHA responsiveness returned by the 42nd day. A second skin test on the 56th day after sensitization did not affect the PHA responsiveness of cells obtained 6 days later.

Peripheral lymphocytes obtained from animals prior to mycobacterial sensitization did not demonstrate any PPD sensitivity as detected by specific antigen-induced transformation. Lymphocyte responsiveness, however, increased up to a net transformation of 12.5% as time after sensitization increased. Skin tests with PPD on days 29 and 56 postsensitization markedly reduced the *in vitro* responsiveness of lymphocytes to 0.5 μg of PPD. This loss of lymphocyte transformation was not evident 2–3 days after skin testing but was significant 6 days after skin

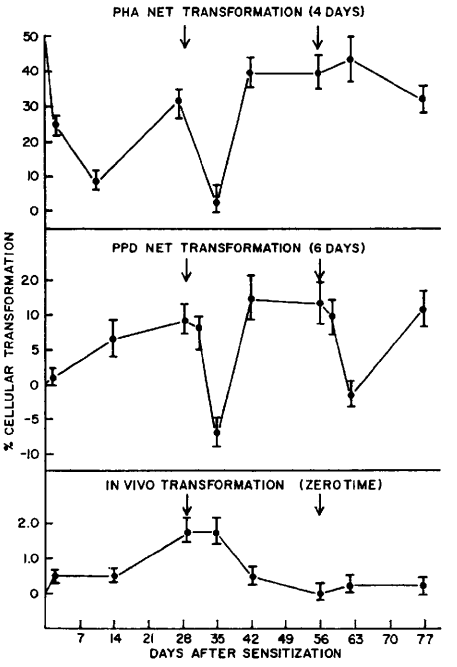

FIG. 2. Response of lymphocytes to *in vitro* PHA and PPD stimulation at various times after guinea pigs were sensitized with *M. tuberculosis*. Net transformation indicates the specific induced cellular transformation—transformation occurring in control cultures. *In vivo* transformation indicates cellular transformation which has occurred *in vivo* and is detected at the start of culture. Band indicates standard error. Arrows indicate time of skin testing with 1 μg of PPD.

TABLE I. NDV Inhibition of PPD-Induced Transformation of Guinea Pig Lymphocytes.

Cultures and treatments[a]	No. of animals[b]	Cell viability (%)[c]	Cellular transformation (%)[d]	Cellular transformation Increase or decrease
No additives	10	75.3 ± 1.9	14.3 ± 2.2	—
0.5 μg of PPD	10	72.3 ± 4.5	23.4 ± 3.0	+64(hs)[e]
1.28 HA units of NDV	10	70.3 ± 3.1	6.7 ± 0.9	<BG[f]
0.128 HA units of NDV	6	72.6 ± 4.4	10.7 ± 1.1	= to BG(ns)
PPD + 1.28 HA units of NDV	10	63.9 ± 2.5	5.3 ± 1.7	<BG
PPD + 0.128 HA units of NDV	6	73.8 ± 3.4	12.9 ± 1.6	—114(hs)

[a] Type of additives (0.1 ml) to 2.4 × 10^6 peripheral leukocytes.

[b] Animals had 4+ skin test reaction at 24 hr following id injection of 1 μg of PPD or 1:2500 dilution of OT.

[c] Average of 20 or 12 cultures of 10 or 16 animals. Value expressed as % viable cells ± standard error (SE).

[d] Average from 10 or 6 animals; 2 cultures/treatment, 2 cell smears/culture, 1000 mononuclear cells counted/smear. Value expresses % blastoid cells ± SE.

[e] p value determined by t test (20); (hs) highly significant, 0.02–0.001; (ns) not significant, 0.05.

[f] BG background. Cellular transformation detected in cultures without additives.

testing (—8.3 and —2% on days 35 and 62, respectively). Values returned to normal levels within the following 1–2 weeks.

The effect of NDV on specific antigen-induced transformation of lymphocytes from guinea pigs is demonstrated in Table I. Optimal concentration and state of NDV needed to inhibit PPD-induced lymphocyte transformation without causing cell death was determined in earlier trials, and found to be dose dependent. Data on the virus dose response showed that live virus concentrations greater than 0.128 HA units depressed cell viability and caused a suppression of cellular transformation in nonstimulated cultures. Ultraviolet-irradiated virus was less effective and not consistent in suppressing the PPD-induced lymphocyte transformation. Table I shows that PPD caused a significant 64% increase in transformed cells when compared to nonstimulated lymphocyte cultures. 0.128 HA units of NDV completely inhibited this PPD-induced transformation without affecting the viability of the lymphocytes and without affecting the background transformation of non-PPD-stimulated cultures.

Discussion. Sensitized peripheral blood lymphocytes from guinea pigs immunized against Freund's complete adjuvant have been shown to be responsive to PPD stimulation *in vitro* (16–17). The present studies show that cells obtained from guinea pigs sensitized to heat-killed *M. tuberculosis* $H_{37}RV$ undergo maximumn PPD-induced blast transformation on the sixth day of culture. This agrees well with previous studies; however, the 0.5 μg of PPD required to obtain maximum transformation was much less than the 10 μg of PPD/culture required in other studies (17). Such a difference may be attributed to slight differences in culture technique or to the method of sensitization.

Present studies show that caution must be exercised when evaluating the *in vitro* responsiveness of peripheral blood lymphocytes from sensitized animals. Specific antigen response increased during the 30 days following sensitization with heat-killed mycobacterium. This increased responsiveness may indicate the development of a population of peripheral blood lymphocytes sensitive to PPD. Skin testing, however, produced a decided decrease in *in vitro* cellular responsiveness to PPD. The decreased responsiveness was not evident immediately after skin testing, but was most pronounced 6 days later and returned to normal within 2 weeks after skin testing. The cause of such a temporary specific antigen-induced unresponsiveness is unknown, but speculations include (i) a removal of PPD-sensitized cells from the peripheral blood compartment, or (ii) a

change in responsiveness to PPD which perhaps could be detected by a different concentation of PPD stimulant *in vitro*.

Sensitization and skin testing with an antigen also affected the responsiveness of peripheral blood lymphocytes to PHA stimulation *in vitro*. Blast transformation following PHA stimulation *in vitro* is regarded as an indicator of cellular responsiveness and immunological competency (4). However, PHA-induced blast transformation of lymphocytes decreased from 47 to 8% 14 days after sensitization with mycobacterium and from 35 to 7% 6 days after skin testing tuberculin sensitive guinea pigs with PPD. A comparable unresponsiveness to PHA has been reported in mouse spleen cells obtained 5 days after the animals were inejcted with casein or Freund's complete adjuvant (18). This unresponsiveness was attributed to a prior *in vivo* commitment of PHA responsive cells to the casein and adjuvant. Such a possibility may be supported by data that show the number of lymphocytes recognized as blastoid cells in the peripheral blood lymphocyte population increased from 0 to 1.8% following sensitization.

These changes in cellular responsiveness to antigens and mitogens show that time after immunization or sensitization is an important factor when comparing normal responsiveness of cells to *in vitro* stimulants and when determining the effect of inhibiting agents such as drugs and microorganisms. The prior history of antigenic stimulation *in vivo* should be known and considered when judging the total cellular response against any *in vitro* stimulation.

Previous studies have shown that peripheral lymphocytes obtained from normal and from tuberculin positive human beings are rendered unresponsive to stimulation by PHA, pokeweed mitogen, and antigens if infected with rubella virus, NDV, or measles virus (9, 10). It was suggested that this unresponsiveness was due to virus altering either the receptor sites at the cell membrane or the intracellular metabolic pathways of the cell (9, 19). The present studies show that NDV is capable of causing a similar inhibition of specific PPD-induced transformation of lymphocytes obtained from guinea pigs sensitized by *Mycobacterium tuberculosis*. Consequently, an animal model has been established which will allow detailed investigations of the virus–lymphocyte relationship.

Summary. Blast cell transformation *in vitro* of lymphocytes obtained from the peripheral blood of guinea pigs after sensitization to mycobacterial antigen was shown to increase after PPD stimulation. However, the cellular activity of lymphocytes was influenced by recent sensitization or immunization of the animals. The blastogenic response of guinea pig lymphocytes to the mitogen PHA was decreased following both the initial tuberculin sensitization and skin testing with PPD. Lymphocytes from tuberculin-sensitized animals demonstrated a fall in responsiveness to *in vitro* PPD stimulation after the animals were skin tested.

Furthermore, this study shows that *in vitro* NDV infection of sensitized lymphocytes prevents the cells from undergoing PPD-induced transformation. This confirms earlier work on viral-induced lymphocyte unresponsiveness and establishes the guinea pig as an animal model for investigating the virus–lymphocyte relationship.

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Received July 15, 1970. P.S.E.B.M., 1971, Vol. 136.