IN VITRO VIRUS IMPAIRED PERIPHERAL BLOOD LEUKOCYTES RESPONSES IN GUINEA PIGS

by

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A Thesis Submitted to the Faculty of the

DEPARTMENT OF MICROBIOLOGY AND MEDICAL TECHNOLOGY

In Partial Fulfillment of the Requirements
For the Degree of

MASTER OF SCIENCE
WITH A MAJOR IN MICROBIOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

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ACKNOWLEDGMENTS

The author extends sincere thanks to Dr. George B. Olson, his academic adviser, for his guidance, kind assistance, and encouragement throughout the course of this investigation.

The author is grateful to Dr. R. J. Janssen for the supply of Newcastle disease virus and to Mr. Clairmont G. Drube for his invaluable help and technical assistance.

Appreciation is extended to Mrs. D. Guthrie, Mr. William L. Wallen, Mr. Jack H. Dean, and Mr. David P. Lilley for their help and cooperation.

Finally, acknowledgment is due the University of Arizona Institutional Grant for financial support of this investigation.

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ABSTRACT

This investigation has shown the guinea pig to be a useful animal model for investigating the immunological competency of virus-infected lymphocytes.

Variations on positive *in vitro* tuberculin purified protein derivative (PPD) induced responsiveness of *Mycobacterium*-sensitized leukocytes from guinea pigs were recorded. A maximum net transformation of 12.2% blastoid cells was obtained with 0.5 μg PPD and a culture period of six days. However, no differences were observed in specific responses of leukocytes to Old Tuberculin stimulation as a function of the culture period.

Optimal concentration of live Newcastle disease virus (NDV) needed to impair specific PPD stimulation of leukocytes without causing cell death was equivalent to 1.28×10^{-1} hemagglutination units. This concentration of live NDV inhibited 97 to 100% of the PPD-induced transformation of leukocytes. Ultraviolet-irradiated NDV, in general, caused less inhibition of specific stimulation of leukocytes than live NDV.

Moreover, results indicate that *Mycobacterium*-sensitized lymphocytes obtained from recently skin-tested animals were unresponsive to *in vitro* stimulation with PPD and phytohemagglutinin. Possible reasons for this *in vitro* "anergy" are discussed.

INTRODUCTION

The phenomenon of *in vitro* phytohemagglutinin (PHA) induced transformation of lymphocytes was first demonstrated by Nowell (1) in 1960. He showed that peripheral blood leukocytes of human beings cultured with PHA (2), an extract of kidney beans (*Phaseolus vulgaris*), underwent marked morphological changes and increased mitotic division. Later, Hastings et al. (3) demonstrated that the cells being transformed to blastoid cells were small lymphocytes.

The morphological, staining and biochemical characteristics of transformed blastoid cells have been studied by many investigators (4-14). When a small peripheral blood lymphocyte is stimulated with a general mitogen such as PHA, the cell responds by an increase in cell size, a decrease in nuclear-cytoplasm size ratio, presence of one or more prominent nucleoli in the nucleus, presence of acidophilic granules, and the development of a well-defined cytoplasmic membrane (4-7). Transformed cells, when stained with Wright or Giemsa stain, demonstrate an eccentrically located nucleus with a heterochromatic nucleoplasm and a basophilic cytoplasm which may contain azurophilic granules and small, well-defined vacuoles (4-7). Biochemical studies, using specific radioisotopes, show that PHA stimulation induces a marked increase in synthesis of cellular protein (8), ribonucleic acid (9, 10) deoxyribonucleic acid (11), and specific enzymes (12). Such a highly active transformed cell is capable of undergoing several mitotic divisions (1, 13, 14).

The degree of lymphocyte transformation is ascertained by several methods. One method is a differential count of the number of blastoid cells per 1000 mononuclear cells of culture (7, 15, 16). In this method, the number of transformed cells is expressed as percentage of transformation of small lymphocytes to blast type cells (16). A second method is a differential count of mitotic figures per 1000 mononuclear cells of culture (17). A third method is to pulse label the culture with radioisotopes such as tritiated thymidine which is incorporated into the DNA of the transformed blast cells (18, 19). The degree of incorporation of tritiated thymidine is subsequently detected by radioautography (20) or liquid scintillation counting procedures (18, 19).

Peripheral leukocytes of most animals have been subjected to PHA stimulation in vitro. Comprehensive evaluation, however, has been restricted to peripheral leukocytes of human beings. Few reports have been concerned with leukocytes of the guinea pig. Pearmain and Lycette (21) reported blastogenesis of peripheral lymphocytes of guinea pigs stimulated with PHA. Aspergren and Rorsman (22) and Zweiman, Besdine, and Hildreth (23) reported marked mitotic activity in PHA-stimulated cultures of guinea pig peripheral blood leukocytes. Marshall and Roberts (24), however, were not able to obtain PHA-induced stimulation of peripheral lymphocytes of guinea pigs.

Positive results were also demonstrated by other investigators (25-28). Knight et al. (25) showed blastogenesis in cultures of 0.5×10^6 guinea pig peripheral blood lymphocytes stimulated with 0.03 ml of

phytohemagglutinin-M. Cultures contained 3 ml of Eagle's medium with 10% tryptose, penicillin, and streptomycin and were cultured for a 24- or 48-hr period. Sabesin (26) showed positive PHA stimulation of guinea pig peripheral lymphocytes when cultured for 72 hours in 4 ml of Minimum Essential Medium containing 1% glutamine and 20% fetal calf serum or 20% rabbit serum. Oppenheim, Wolstencroft, and Gell (27), using 0.1 ml of PHA-M per 6 x 10^6 guinea pig peripheral leukocytes in 3 ml of NCTC 109 containing 40% heat inactivated calf serum, showed blastogenesis on the fifth and seventh day of culture. Oppenheim (28) was also able to demonstrate positive responses in 6-15 x 10^6 guinea pig peripheral leukocytes stimulated with PHA after 92 hours of culture.

Concomitant with the increased findings of PHA-induced transformation was the discovery that peripheral leukocytes would respond to *in vitro* stimulation with specific antigens with which the individual had been sensitized. However, blastogenesis occurs more slowly and to a lesser extent in specific antigen-stimulated culture than in PHA cultures, and the transformation is less affected by changes in concentration of the stimulant when used within nontoxic ranges (29).

Several early papers reported conflicting results when peripheral leukocytes of tuberculin-sensitized guinea pigs were stimulated with purified protein derivative (PPD) of *Mycobacterium tuberculosis in vitro*. Some attempts to demonstrate tuberculin (22) and tuberculin PPD (25) specific stimulation of cells from these animals were unsuccessful. Positive results seem to depend upon improved culture conditions as shown in more detailed experimentations by Oppenheim et al. (27) and

Oppenheim (28). These authors obtained positive transformation responses on the fourth to seventh days of culture when using 10 μg per ml of tuberculin PPD or 0.1 ml of 1:10 dilution of Old Tuberculin (OT) per 6 x 10^6 sensitized guinea pig peripheral blood leukocytes in 3 ml culture.

In general, stimulation of peripheral blood leukocytes from healthy human beings demonstrated a significant-to-high degree of PHA and specific antigen-induced in vitro transformation. However, leukocytes from patients with such diseases as Hodgkin's disease (5, 30), chronic lymphatic leukemia (6), sarcoidosis (5, 31), ataxia telangiectasia (32), agammaglobulinemia (9), thymic aplasia (5, 33), uremia (34), and lymphocytes from babies with congenital rubella (16, 35, 36) showed varying degrees of unresponsiveness to such in vitro stimulation. In addition, these patients usually demonstrate varying degrees of decreased cellular immunity as determined by (a) delayed skin-test responses to various common antigens, (b) rejection of skin allografts, and (c) susceptibility to infection (5, 6, 9, 16, 30-34). Impaired in vitro lymphocyte transformation has also been noted in post-operative situations (37) and with the use of chemotherapeutic drugs which are known to inhibit the antibody response (38).

These observations prompted Hersh and Oppenheim (5) to correlate aspects of decreased cellular immunity and negative in vitro leukocyte transformation, and to suggest that a negative in vitro response to PHA or specific antigen was indicative of an impaired immunological competent cell or condition.

Olson et al. (36) showed that rubella virus and Newcastle disease virus infections of peripheral leukocytes from human beings would impair the ability of these cells to respond to *in vitro* stimulation with general mitogens (PHA and pokeweed mitogen) and specific antigens (diphtheria toxoid and tetanus toxoid). Smithwick and Berkovich (39) also revealed that measles virus could inhibit the *in vitro* PPD-induced transformation of peripheral lymphocytes from mantoux positive children. Olson et al.(36) suggest that some viruses are capable of invading and producing a temporary or permanent unresponsive state in lymphocytes. As such, virus infections could be the cause of decreased immunologic function in certain disease states. The reasoning for such belief is supported by the observations that in certain viral diseases such as measles (40) and influenza (41) a state of "anergy" to tuberculin has been observed.

The purpose of this investigation was to establish the guinea pig as an animal model in order to ascertain the effect of viral infection on peripheral blood leukocytes. Peripheral leukocytes from tuberculin-sensitized guinea pigs were infected *in vitro* with Newcastle disease virus. The effects of viral infection were determined by the ability of the infected cells to undergo blastogenesis and their ability to survive following tuberculin PPD stimulation *in vitro*.

MATERIALS AND METHODS

Animals

Outbred albino male and female guinea pigs of the Rockefeller strain, propagated in the Microbiology Department and weighing 700 to 1000 grams, were employed as donors of peripheral blood leukocytes throughout this study. They were all housed in separate cages in an environment where temperature (74 F $\pm$ 2 F) and relative humidity (50% $\pm$ 5%) were controlled. They were fed Purina guinea pig chow and tap water supplemented with 0.3% ascorbic acid (Merck and Company, Inc., Rhino, New Jersey) ad libitum. Cabbage was provided daily.

Guinea pigs were sensitized to tuberculin by subcutaneous injection in the back of the neck with heat-killed (100 C for one hour) Mycobacterium tuberculosis, H37RV strain, suspended in paraffin oil mixed with melted vaseline (42). Each guinea pig received 0.75 mg of the dried tubercle bacilli in a total inoculum of 1 ml injected into five sites, 0.2 ml per site.

Skin tests were performed on shaved sites of the flanks of the guinea pigs by injecting 0.1 ml containing 1 µg PPD or 1:2500 dilution of OT, intracutaneously. The diameter of induration-associated erythema of each dermal reaction was read and scored arbitrarily at 24 hours after skin testing, using the following scheme:

Score	Skin Reaction Diameter (mm) of Induration-associated Erythema
-	5.0
1^+	5- 7
2^+	8-10
3^+	11-13
4^+	>13

Preparation of Reagents

Phytohemagglutinin (PHA-M, DIFCO Laboratories, Detroit, Michigan, U.S.A., Cont. 532118) was reconstituted with 5 ml saline (0.15M NaCl) and was used in 0.1 ml amount per selected culture.

Tuberculin purified protein derivative (Tuberculin PPD, Parke, Davis and Company, Detroit) lot #984248A was reconstituted with buffered diluent and diluted to varying concentrations with saline (0.1M NaCl). It was used as a specific antigen in quantities ranging from 0.05 μg to 5.0 μg in 0.1 ml volume per culture.

Old Tuberculin (OT, Eli Lilly and Company, Indianapolis, Ind., V-802, control 2L P41B) was diluted to $1:10^{-3}$ to $1:10^{-5}$ with saline and used in 0.1 ml volume per culture. These amounts correspond to 0.1 mg to 0.001 mg of the National Institute of Health Reference standard for OT, respectively.

Newcastle disease virus of Roakin strain (NDV, Cat. No. V-326-001-000, prepared by Chas. Pfizer and Company, Inc., and obtained from Dr. Robert J. Janssen, University of Arizona) was used in concentration of 1.28×10^{-1} to 128 hemagglutination units per selected culture tubes per 0.1 ml volume. The virus stock had been passaged 24 times in the allantoic sac of embryonated chick eggs.

The virus stock was prepared according to the method of Henle and Hilleman (43) by injecting 0.2 ml of diluted (1:100) NDV suspension in allantoic fluid into the allantoic sac of 11-day-old embryonated chicken eggs. After incubation at 35 to 36 C for 48 hours in a humidified atmosphere, moribound embryos were refrigerated overnight and the NDV-infected allantoic fluids were removed aseptically, pooled, and stored in a Revco freezer at -65 C.

The virus was purified by a modified method of Wheelock (44). Infected allantoic fluid was clarified by centrifugation at 1400 x g for one hour. Supernatant fluid was centrifuged at 83,000 to 122,000 x g for 2½ hours in a Beckman Spinco Ultracentrifuge. Pellets were suspended in half the original volume of Minimum Essential Medium (MEM, Grand Island Biological Company, Grand Island, N. Y.) containing 100 units/ml of penicillin and 100 µg/ml of streptomycin (Eli Lilly and Co., Indianapolis, Ind.) and stored in a Revco freezer at -65 C in appropriate small volumes.

The concentration of virus was determined by the hemagglutination procedure (HA). Twofold dilutions (from 1:10 to 1:2560) of NDV suspension were made in 1/15 M phosphate buffered saline (pH 7.2, 0.15 M NaCl) in 0.5 ml amounts; 0.5 ml of 0.5% chicken erythrocytes was added to each tube. The hemagglutination pattern was read after one hour of incubation at room temperature. The same NDV stock was used for all experimentations.

Ultraviolet-irradiated (UVI) NDV was obtained by a modified method of Olson et al. (36). One to two ml of stock virus was irradiated for 10 minutes at a distance of 8 inches from an ultraviolet lamp

(2500-2600 Å) to destroy all or part of its infectivity. Selected concentrations of the live and UVI virus were used in 0.1 ml volume per selected culture.

Anti-NDV serum was prepared by a modified method of Wheelock and Tamm (45). Male rabbits, weighing 3-5 kilograms, were injected twice, two weeks apart, with UVI-NDV suspension (5 ml per injection) into the marginal ear veins. Rabbits were test bled on the 29th day after the first injection. Hemagglutination inhibition (HI) titers of the antisera were determined using 4 HA units of live NDV in 0.25 ml, varying dilutions of heat-inactivated test antiserum in 0.25 ml volumes and 0.5 ml of 0.5% chicken erythrocytes per test tube. Phosphate buffered saline (pH 7.2) was used as a diluent. Thirty ml of blood was taken four times from each rabbit by ear vein puncture at two-week intervals and stored at -20 C. All antisera had an HI titer of 160.

Procurement of Leukocytes

Peripheral blood leukocytes were obtained from tuberculin-sensitized and normal guinea pigs. Heparinized blood samples (10 units of sodium heparin, U.S.P., Rabin Winters Pharmaceuticals, El Segundo, Calif., per ml of blood) were obtained from guinea pigs by cardiac puncture without anesthesia. Peripheral leukocytes were separated from the erythrocytes by the method of Hullinger and Blazkvec (46). Leukocytes separated by this method were centrifuged at 225 x g for 15 minutes. Cell button was resuspended and washed twice with Earle's Balanced Salt Solution containing 30 units of heparin per ml, 100

units/ml of penicillin, 100 µg/ml of streptomycin and 2.2 g/l of sodium bicarbonate. After the final wash, leukocytes were resuspended in 2 ml of Earle's Balanced Salt Solution (Earle's BSS). Cell viability was assayed by the dye exclusion method (47) using 0.2% trypan blue stain (Grand Island Biological Company), as a final concentration. Total leukocyte counts were done by a standard method (48). Cell suspensions prepared by this procedure contained about 60% mononuclear cells.

Culture Conditions

Cell concentration was adjusted to 0.8×10^6 viable leukocytes per ml in McCoy 5-A medium (modified) (Grand Island Biological Company, Cat. No. H-15) containing 100 units/ml of penicillin and 100 µg/ml of streptomycin, 30 units/ml of heparin, 20% fetal calf serum (Grand Island Biological Company), and about 1.76 g/l of sodium bicarbonate ($NaHCO_3$, J. T. Baker Chemical Company, Phillysburg, N. J.). Cultures containing 3 ml each were prepared in 125 x 15 mm round-bottomed "Thro-Away" culture tubes (Bellco Glass Company, Inc., Vineland, N. J.) with Morton closures. Selected cultures received PHA or specific antigen and/or virus preparation in 0.1 ml volume. Cultures were placed in a roller drum (72° rotation/min) and incubated at 37 C in a 5% CO_2 humidified atmosphere for 4, 5, 6, or 7 days.

Evaluation of Cultures

Cultures stimulated with PHA or specific antigens and the appropriate control cultures were terminated at various times between the fourth and seventh day. Cultures were centrifuged at 150 x g for 5

minutes. Sedimented leukocytes were resuspended and washed twice with Earle's BSS containing no calcium chloride. After a final wash, cells were resuspended in about 0.2 ml of Earle's BSS.

Cell viability was determined by the dye-exclusion procedure using trypan blue dye (47). Single cell preparations of smears were prepared in duplicate per culture on two separate microscope slides and stained with Giemsa.

Responses of peripheral blood leukocytes from tuberculin-sensitized guinea pigs in cultures stimulated with PHA or specific antigen as compared to nonstimulated cultures were determined by a differential count of the number of blastoid cells in 1000 mononuclear cells per smear (7, 15, 16). Macrophages were not included in the mononuclear count. The number of blastoid cells was expressed as the percent of transformed blastoid cells in the mononuclear cell population (16).

RESULTS

Specific responses of peripheral blood leukocytes from Mycobacterium-sensitized guinea pigs to in vitro PPD or OT stimulation were investigated. Three experimental runs were made using one guinea pig per experiment. All guinea pigs had an average skin reaction of 2^{+}. Both skin test antigens provoked similar skin reactions in guinea pigs employed in this and subsequent experiments. A different count at the start of the experiment showed that 60% of the peripheral blood leukocytes were mononuclear-type cells and 2% of these mononuclear cells possessed blast-like characteristics.

Variations in the positive PPD responses of leukocytes from sensitized guinea pigs are presented in Table 1. Maximum PPD-induced net transformation in 2.4×10^{6} peripheral leukocytes was $12.2\% \pm 4.2$ as shown in Fig. 1. This response represents a 299% increase in transformation over the corresponding control transformation of $4.2\% \pm 2.0$. This maximal stimulation was obtained with 0.5 μg PPD and a culture time of six days.

The lack of differences in specific responses of leukocytes from sensitized guinea pigs to OT stimulation as a function of culture period is shown in Table 2. No significant correlation between concentration of OT and transformation was observed during the three-day culture periods. However, responses observed in cultures containing

Table 1. *In vitro* PPD-induced transformation in peripheral blood leukocytes of *Mycobacterium*-sensitized guinea pigs.

Amount of PPD (0.1 ml) Added to 2.4×10^6 Leukocytes	Number of Guinea Pigs[b]	Transformation[a] (Net Percent)		
		Culture Period (Days)		
		5	6	7
0.05 µg	3	6.2[c] ± 2.3[d]	5.2 ± 0.8	5.5 ± 1.0
0.5 µg	3	6.5 ± 2.4	12.2 ± 2.0	10.6 ± 2.1
5.0 µg	3	5.8 ± 2.5	7.1 ± 0.6	6.9 ± 3.1

a. Net percent transformation (% transformation in cultures stimulated with PPD minus % transformation in corresponding culture without PPD). Transformations in cultures without PPD for days 5, 6, and 7 of culture periods were 5.2% ± 2.5, 4.2% ± 2.0 and 7.7% ± 1.8, respectively.

b. Guinea pigs had an average dermal reaction of 2+.

c. Average from three guinea pigs, one culture per treatment, one cell preparation or smear per culture.

d. Standard error.

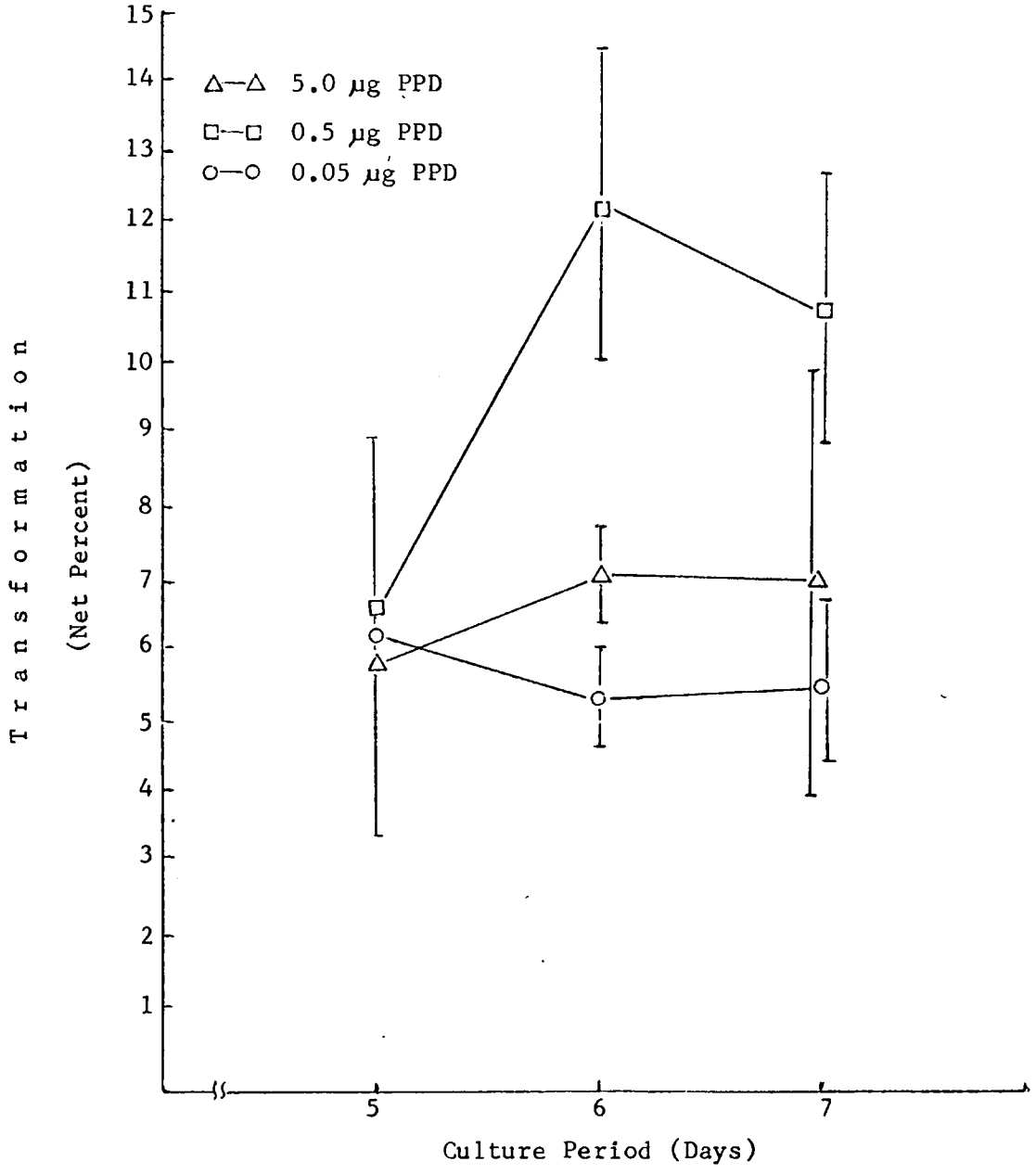

Fig. 1. PPD Dose Response of Leukocytes From Mycobacterium-sensitized Guinea Pigs as a Function of Time in Culture.

Graph lines join the mean of net % transformations presented in Table 1.

Table 2. *In vitro* OT-induced transformation in peripheral blood leukocytes of *Mycobacterium*-sensitized guinea pigs.

Amount of OT (0.1 ml) Added to 2.4×10^6 Leukocytes	Number of Guinea Pigs[b]	Transformation[a] (Net Percent) Culture Period (Days)		
		5	6	7
0.001 mg	3	3.7[c] ± 1.7[d]	3.1 ± 1.2	3.5 ± 2.6
0.01 mg	3	5.2 ± 1.4	5.2 ± 2.1	8.6 ± 2.1
0.1 mg	3	8.9 ± 4.6	7.6 ± 3.4	7.8 ± 3.6

a, b, c, and d. Same as Table 1.

0.01 mg or 0.1 mg of OT per culture were higher than responses observed in cultures containing 0.001 mg OT per culture. The highest degree of transformation (8.9% $\pm$ 4.8) was recorded with 0.1 mg OT at the fifth day of culture.

Consequently, 0.5 µg PPD per 2.4 x 10^6 peripheral leukocytes and a culture period of six days were selected as experimental conditions for subsequent experiments.

Optimal concentration and state of NDV needed to inhibit PPD-induced leukocyte transformation without causing cell death was investigated. Guinea pigs used as donors of leukocytes for these experiments had an average skin reaction of 4+. Peripheral leukocytes used in these cultures were 60% mononuclear cells and less than 2% of these were recognized as blastoid cells.

Table 3 shows 18.0% blastoid cells in PPD-stimulated cultures as compared to 10.7% blastoid cells in nonstimulated cultures. This represents a net increase of 68%. Impairment of transformation of leukocytes to PPD stimulation was shown to be virus dose dependent. Live NDV at 1 x 10^{-3} dilution (1.28 x 10^{-1} HA units/0.1 ml) induced 97% inhibition of the transformation of leukocytes while NDV at 1 x 10^{-2} and lower dilutions caused complete inhibition. In general, UVI-NDV produced less inhibition of PPD-induced leukocyte transformation than live NDV. For example, UVI-NDV at 1 x 10^{-3} dilution inhibited 32% of PPD-induced leukocyte transformation while live NDV at 1 x 10^{-3} dilution inhibited 97% of the cellular transformation.

Table 3. Inhibition of PPD-induced transformation of guinea pig leukocytes as a function of NDV concentration.

Additives to 2.4×10^6 Leukocytes (0.1 ml)	Number of Guinea Pigs[c]	Live NDV		Ultraviolet-irradiated-NDV	
		Transformation (Percent)	Increase[a] or Decrease[b] of Transformation (Percent)	Transformation (Percent)	Increase or Decrease of Transformation (Percent)
No additive	8	10.7[d] ± 1.6	-	10.7 ± 1.6	-
0.5 μg PPD	8	18.0 ± 2.4	+ 68 (hs)[e]	18.0 ± 2.4	+ 68 (hs)
Stock NDV[f]	6	2.5 ± 2.2	< BG	10.4 ± 2.3	= BG
1×10^{-2} dil. of stock NDV	4	7.8 ± 1.8	< BG	11.4 ± 3.4	= BG
1×10^{-3} dil. of stock NDV	6	10.7 ± 1.1	= BG	N.D.	N.D.
0.5 μg PPD + stock NDV	8	2.1 ± 1.6	<-100 (hs)	11.3 ± 2.3	- 92 (hs)
0.5 μg PPD + 1×10^{-1} dil. of stock NDV	8	6.2 ± 1.3	<-100 (hs)	12.0 ± 1.9	- 82 (hs)

Table 3.--Continued

0.5 μg PPD + 1 x 10^{-2} dil. of stock NDV	8	7.1 ± 1.0	<-100 (hs)	13.8 ± 2.0	- 58 (hs)
0.5 μg PPD + 1 x 10^{-3} dil. of stock NDV	8	10.9 ± 1.5	- 97 (hs)	15.7 ± 2.4	- 32 (ns)

N.D.- Not done.

BG - Background

a. Increase (%) = $\frac{\text{\% transformation for PPD - \% transformation for no additive}}{\text{\% transformation for no additive}} \times 100$.

b. Inhibition (%) = $\frac{\text{\% transformation for PPD - \% transformation for PPD + NDV}}{\text{\% transformationfor PPD - \% transformation for no additive}} \times 100$.

c. Guinea pigs had an average dermal reaction of 4+.

d. Average from eight guinea pigs; one culture per treatment, two cell preparations or smears per culture.

e. P value as determined by t-test (49) 0.05 not significant (ns), 0.05-0.02 significant (s), and 0.02-0.001 highly significant (hs).

f. Stock NDV had an HA titer of 640 per 0.5 ml.

Observations on the effect of NDV on survival of peripheral leukocytes in PPD-stimulated and nonstimulated cultures are presented in Table 4. Cell viability, at the beginning of the culture period in these and other experiments was approximately 100%. On the sixth day of the culture period, cell viability as determined by exclusion of trypan blue was 36 to 77%. Cell viability was slightly higher in six-day cultures infected with UVI-NDV than in those infected with live NDV. Cell cultures containing both PPD and virus demonstrated a synergistic phenomenon between the antigen and the virus as detected by a reduced cell viability.

No significant differences ($P > 0.1$) were observed in the percentage of mononuclear cells ($60\% \pm 4$) in six days stimulated or nonstimulated cultures as compared to zero time cultures. However, it appeared that six-day cultures containing PPD and NDV had a slightly higher mononuclear cell count than cultures with no additive, NDV or PPD.

Live NDV at 1×10^{-3} dilution was selected as the most desirable dilution of virus for subsequent experiments. This dilution of NDV was selected because it caused a highly significant ($P < 0.01$) inhibition of PPD-induced transformation of leukocytes, it did not affect the viability of leukocytes, and it did not affect the background transformation of non-PPD stimulated cultures.

Data depicting the inhibition of PPD-induced transformation in leukocytes from sensitized guinea pigs by NDV are shown in Table 5. Leukocyte-donor guinea pigs had an average skin reaction of 4+. Net

Table 4. Survival of peripheral leukocytes in NDV-infected cell cultures.

Additives to 2.4×10^6 Leukocytes (0.1 ml)	Number of Guinea Pigs	Cell Viability[a] (Percent)	
		Live NDV	UVI - NDV
No additive	8	76.6[b] ± 3.5	76.6 ± 3.5
0.5 μg PPD	8	64.6 ± 8.1	64.6 ± 8.1
Stock NDV	6	56.3 ± 9.7	73.7 ± 5.7
1×10^{-2} dil. of stock NDV	4	68.0 ± 8.0	72.8 ± 4.6
1×10^{-3} dil. of stock NDV	6	72.6 ± 4.4	N.D.
0.5 μg PPD + stock NDV	8	36.6 ± 4.6	49.0 ± 1.8
0.5 μg PPD + 1×10^{-1} dil. of stock NDV	8	47.0 ± 5.8	62.0 ± 7.5
0.5 μg PPD + 1×10^{-2} dil. of stock NDV	8	55.9 ± 8.0	63.0 ± 7.0
0.5 μg PPD + 1×10^{-3} dil. of stock NDV	8	69.1 ± 5.8	68.4 ± 5.3

N.D. - Not done.

a. Cell viability was determined by trypan blue dye exclusion method.

b. Average of cell viability from cultures of eight animals.

Table 5. In vitro inhibition of PPD-induced transformation of guinea pig leukocytes by NDV.

Additives to 2.4×10^6 Leukocytes (0.1 ml)	Number of Guinea Pigs[c]	Transformation (Percent)	Increase[a] or Inhibition[b] of Transformation (Percent)	Cell Viability (Percent)
No additive	10	$14.3^{d} \pm 2.2^{e}$	-	$75.3^{f} \pm 1.9^{e}$
0.5 µg PPD	10	23.4 ± 3.0	+ 64 (hs)	72.3 ± 4.5
1×10^{-2} dil. of stock NDV	10	6.7 ± 0.9	BG	70.3 ± 3.1
0.5 µg PPD + 1×10^{-2} dil. of stock NDV	10	5.3 ± 1.7	- 100 (hs)	63.9 ± 2.5
1×10^{-3} dil. of stock NDV	6	10.7 ± 1.1	= BG	72.6 ± 4.4
0.5 µg PPD + 1×10^{-3} dil. of stock NDV	6	10.0 ± 1.6	- 100 (hs)	73.8 ± 3.4

a. Same as a, Table 3.

b. Same as b, Table 3.

c. Guinea pigs had an average dermal reaction of 4+.

d. Average from 10 guinea pigs; two cultures per treatment, two cell preparations or smears per culture.

e. Standard error.

f. Average of cell viability from 20 cultures of 10 animals.

g. Same as e, Table 3.

BG - Background.

PPD-induced transformation of leukocytes was 9.1%. This is equivalent to a 64% increase of leukocyte transformation in PPD-stimulated cultures. Live NDV at 0.1 ml volumes of 1×10^{-3} or 1×10^{-2} dilutions caused approximately 100% inhibition of PPD-induced transformation of leukocyte cultures. Cell viability was comparable to control cultures in all cases except in cultures with PPD and the higher concentration of live NDV.

Variations in responses of guinea pig peripheral leukocytes to PHA or PPD stimulation after tuberculin sensitization and/or skin testing are shown in Table 6. PHA-induced net transformation of peripheral leukocytes from four normal guinea pigs was 47% after a 92-hour culture period (Fig. 2). This response was significantly affected ($P < 0.01$) following immunization with heat-killed Mycobacterium tuberculosis. At day 2 through day 14 after immunization, peripheral leukocytes failed to respond adequately to PHA stimulation in vitro as depicted by decreased transformation values of 24.9% $\pm$ 3.0 and 8.9% $\pm$ 1.2, respectively. By day 29, peripheral leukocytes had regained their responsiveness to PHA stimulation. Skin testing with 0.1 ml volumes containing either 1 µg PPD or 1:2500 dilution of OT on the 29th day after immunization caused a significant decrease ($P < 0.01$) in leukocytes responsiveness to PHA as detected in leukocytes obtained on the sixth day after skin testing (35th day after immunization). A second skin test applied on the 57th day after immunization did not have a significant effect ($P > 0.05$) on PHA-induced transformation of leukocytes obtained on the sixth day after skin testing.

Table 6. *In vitro* PPD-induced transformation of guinea pig leukocytes as a function of time post immunization and/or skin tests.

Additive to 2.4×10^6 Leukocytes (0.1 ml)	Transformation[a] (Percent) Days Post Immunization[b]									
	0[c]	2	14	29	35	42	56	63	77	110[d]
No additives (zero time)	0.0±0.0[e,f]	0.5±0.0	0.4±0.1	1.8±0.4	1.8±0.4	0.5±0.1	0.0±0.0	0.2±0.1	0.2±0.1	0.3±0.0
No additive (PHA control)	10.3±3.3	8.6±2.0	10.0±0.4	12.4±3.0	21.1±3.7	7.8±0.9	16.1±0.6	13.6±1.5	15.7±0.5	12.5±2.0
Stock PHA	57.7±7.7	33.5±3.9	18.9±2.0	44.8±3.3	18.3±1.2	47.7±6.9	56.0±4.2	59.2±0.3	47.7±6.2	53.5±4.7
No additive (PPD control	22.3±1.5	16.2±3.8	13.1±0.8	24.3±1.6	29.0±2.7	28.3±4.3	35.1±2.9	24.3±2.4	25.6±9.4	15.5±1.4
0.5 µg PPD	22.0±3.1	16.4±3.3	21.0±3.2	33.8±6.3	21.7±1.5	40.8±7.8	37.5±1.7	22.3±2.1	36.6±4.5	21.0±2.1

a. Transformation was determined at the end of 92 hours of culture period for PHA experiments and on the sixth day for PPD experiments.

b. Each of the four guinea pigs was immunized with 0.1 ml containing 0.75 mg heat-killed H37RV strain of *Mycobacterium tuberculosis*. They were skin tested with 0.1 ml containing 1 µg PPD or 1:2500 dilution of OT on the 29th and 57th day after immunization.

c. On day zero four normal guinea pigs (not used subsequently) were employed.

d. On day 110 only one guinea pig, not used before, was employed; skin tested three times; last skin test was applied three days before leukocyte procurement.

e. Average from four guinea pigs; two cultures per treatment; two cell preparations or smears per culture.

f. Standard error.

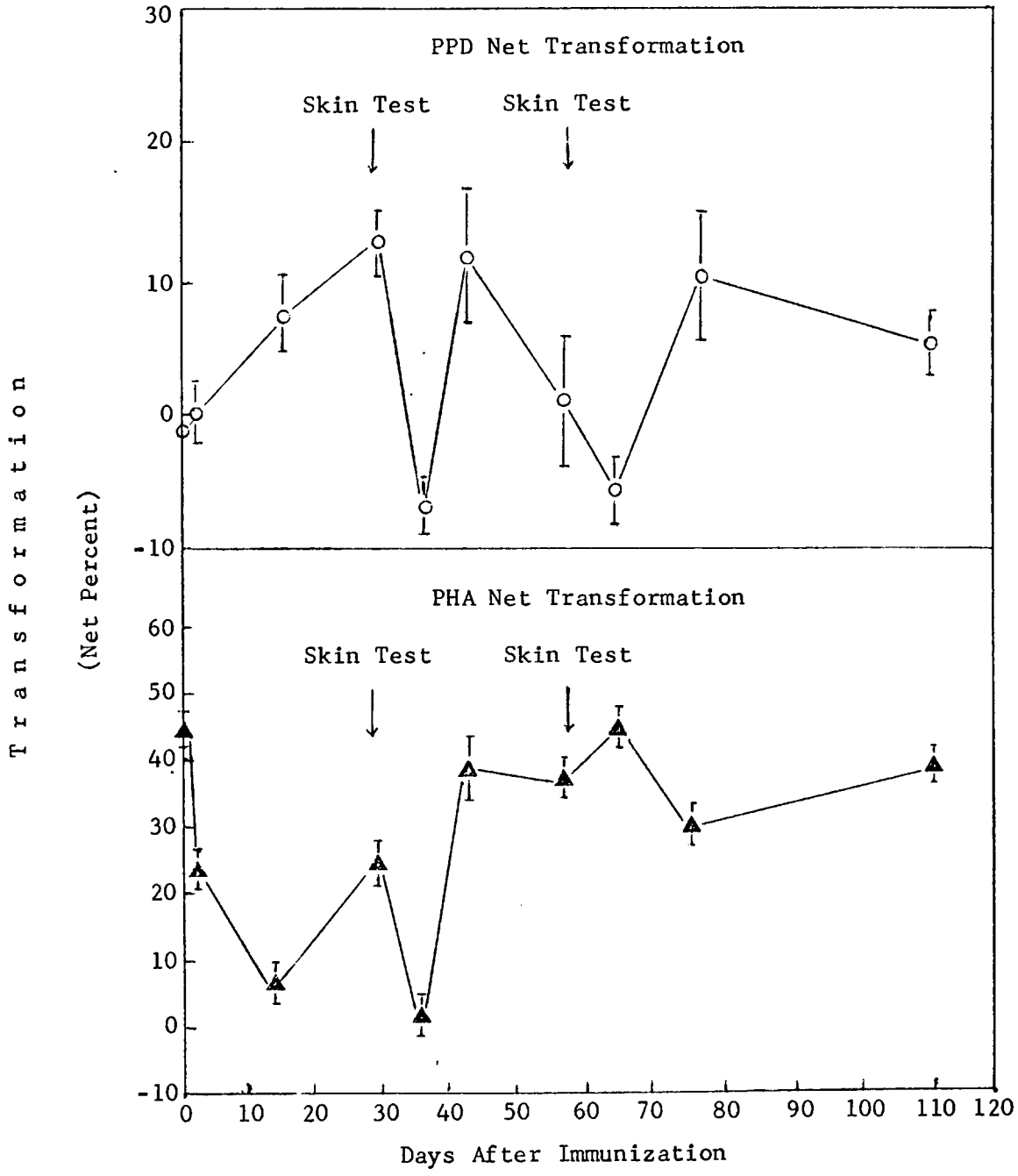

Fig. 2. Relationship of Blastogenesis of Guinea Pigs Peripheral Leukocytes to in vitro PPD and PHA Stimulation After Tuberculin Sensitization and/or Skin Testing.

Transformation of leukocytes from tuberculin-sensitized guinea pigs to PPD stimulation as a function of time is shown in Fig. 2. Peripheral leukocytes from normal animals did not demonstrate any PPD sensitivity as detected by specific antigen-induced net transformation. Leukocyte responsiveness, however, increased up to a net transformation of 14.5% as time after immunization increased. This maximum response was obtained between the 29th and 42nd day post immunization. Skin testing with 0.1 ml volume containing either 1 μg PPD or 1:2500 dilution of OT significantly reduced the *in vitro* PPD-induced transformation of leukocytes procured six days after skin testing.

A differential count of the peripheral leukocytes at zero time of culture showed no significant change in the *in vivo* transformation of leukocytes as time after immunization increased or due to skin testing (Table 6). Only 0.0 to 1.8% of the mononuclear cells were recognized as blastoid cells at the start of each experiment.

Table 6 shows the degree of lymphocyte transformation in non-stimulated four-day and six-day cultures as a function of time after immunization and/or skin testing. *In vitro* transformation in the four-day cultures (non-PHA stimulated control cultures) remained at 9 to 16% blastoid cells throughout the immunization period. The only exception was at the 35th day (six days after the first skin testing) when transformation in control cultures increased to 21.1% $\pm$ 3.7.

In contrast, *in vitro* leukocyte transformation in the six-day cultures (non-PPD stimulated cultures) seemed to increase during the first 56 days of the immunization and skin testing period. This is

evident when comparing the 13 to 16% blastoid cells of the first two weeks against the 24 to 35% transformation obtained in cultures of leukocytes tested between the 29th and 56th days. Values after the 56th day slowly decreased to the previously recorded values of 15%. Values for transformation at zero day of immunization were obtained from four different guinea pigs--not the four animals used in this immunization schedule. Values for transformation at the 110th day after immunization were also obtained from one guinea pig used in the dose-response study of NDV (Table 3).

Two of the guinea pigs used in this time study developed the open lesion type wounds similar to those described by Koch (in 51) after the second skin test was applied. A correlation of the in vitro responsiveness of leukocytes from these animals as compared to guinea pigs without open wounds showed no differences in the degree of blastoid transformation either in nonstimulated or PPD-stimulated cell cultures.

DISCUSSION

Cell culture techniques of peripheral leukocytes from man and animals are widely used to investigate the responses of lymphocytes to general mitogens and specific antigens. These techniques provide possible methods for investigating the immunological status of lymphocyte donors.

The present study provides evidence that specific responses of peripheral leukocytes from sensitized guinea pigs to PPD stimulation *in vitro* are dose- and culture period dependent. Net transformation in 2.4×10^6 peripheral leukocytes induced by 0.5 µg PPD stimulation in six-day cultures ranged from 5 to 15%. Comparison of these values with values of specific transformation of leukocytes of guinea pigs in the literature is impossible since the latter values are expressed in terms of uptake of radioisotope precursors by the leukocytes. However, specific PPD responses in guinea pig leukocytes reported in this study are comparable with published data (2 to 40%) reported for the specific stimulation of leukocytes from human beings in three- to seven-day-old cultures (28).

Net percent transformation in peripheral leukocytes to *in vitro* PPD stimulation did not show a positive correlation with skin test reactions of leukocyte donor guinea pigs. Leukocytes from guinea pigs with a 2+ average skin reaction (Table 1) appeared to respond better to PPD stimulation than leukocytes from guinea pigs with a 4+ average skin reaction (Tables 3 and 5). Closer inspection, however, shows that

the difference in net percent transformation was due only to differences observed in transformation in non-PPD stimulated cultures of leukocytes. Total PPD-induced transformation in leukocytes of guinea pigs showing 2+ skin reaction was 16.4% as compared to 18.0% transformation in leukocytes of guinea pigs with a 4+ skin reaction. However, non-PPD stimulated leukocytes from guinea pigs depicting a 2+ skin reaction had about 4.2% transformation as opposed to 10% transformation in non-PPD stimulated leukocytes of guinea pigs with a 4+ skin reaction. It is interesting to note that these differences in transformation values were observed in non-PPD stimulated six-day cultures and not in transformation values of zero-time cultures.

The lack of differences in specific responses of leukocytes to OT stimulation as a function of culture period is not understood. Perhaps this may be due to the variable chemical nature of OT as compared to tuberculin PPD (50).

This study indicates that OT is less capable of inducing specific *in vitro* transformation of leukocytes from tuberculin-sensitized guinea pigs. This correlates with *in vivo* work which showed that 1 μg PPD and 50 μg OT (1:2500 dilution) provoked similar skin reaction in guinea pigs. This difference is even more apparent in skin testing of human beings where 1 μg PPD was comparable to 200 μg OT (51).

One interpretation of these data suggests that the amount of specific antigens in 100 μg may be below the threshold dose required for *in vitro* stimulation of leukocytes. A second possibility is that the specific antigens in OT may not be able to stimulate cells *in vitro*

as easily as they can in vivo. Moreover, one must be cognizant of the fact that the mechanisms involved in *in vitro* and *in vivo* preparations of antigens and specific stimulation of leukocytes may be different and, as such, *in vivo* and *in vitro* specific responses may not be comparable.

Live NDV at dilutions of 1×10^{0} to 1×10^{-3} have been shown to cause approximately 100% inhibition of PPD-induced transformation. However, it was only 1×10^{-3} dilution of live NDV that inhibited about 100% of PPD responses without causing cell death. In addition to the unattenuated NDV, rubella and measlesviruses have also been shown to induce unresponsive states in peripheral lymphocytes of human beings following *in vitro* specific and nonspecific stimulation (36, 39). It was suggested that the possible mechanisms of viral inhibition were due to 1) alteration of receptor sites at cell membrane surface or 2) alteration of the cellular metabolic pathways by intracellular virus (16).

Live virus can be inactivated by UV irradiation without changing certain biochemical properties (52). Olson et al. (36) have shown that UVI-rubella virus did not inhibit PHA-induced transformation of human leukocytes as did the live rubella virus. Based partly on this data, they hypothesized that viral-induced unresponsiveness was due to a viral infection which gained control of the biochemical events within a lymphocyte. Contrary to this finding, the present study showed that UVI-NDV was still capable of inhibiting PPD-induced transformation of guinea pig leukocytes. However, there was some reduction in the inhibitory capacity of UVI-NDV as compared to live NDV. This promotes

the question of whether or not infectious virus is required to cause complete inhibition of leukocyte specific responses.

There are several factors to consider when comparing the discrepancy of data obtained from the NDV and rubella experiments. One factor is multiplicity reactivation (MR) (52, 53, 54). Rubella virus is not subject to MR following UV irradiation (36). Moreover, studies of MR with NDV reveal a controversy. Barry (55), using the same methods that he developed for influenza, found no evidence of MR with NDV California 11914. Drake (56), however, demonstrated a small amount of MR with L-Kansas 1948 and heat-resistant Beaudette strains of NDV in fresh allantoic fluid. Recent work by Kirvaitis and Simon (57) also demonstrated strong MR with Beaudette strain of NDV in chick embryo cell cultures.

A second possibility which may explain the discrepancy of the data is that an insufficient amount of UV irradiation was used. Perhaps the predominant wavelength of UV irradiation or time of UV-irradiation exposure was not optimal for maximal inactivation of NDV.

A third possible reason includes the presence of virus clumps. Clumping reduces the effect of UV irradiation on certain virus particles. Clumps also increase the chance of MR as indicated by the work of Kirvaitis and Simon (57) where they showed that clumping of NDV following UV irradiation increased infectivity by more than 50-fold.

Peripheral leukocytes from normal guinea pigs stimulated *in vitro* with PHA showed about 47% net transformation of small lymphocytes to blastoid cells. However, tuberculin sensitization *in vivo*

and the first skin-testing after sensitization caused a significant reduction in PHA-induced transformation of leukocytes, *in vitro*. This unresponsiveness might be attributed to a tuberculin-induced alteration in the relative numbers of PHA responsive and non-PHA responsive cells. *Mycobacterium tuberculosis* is composed of various antigens; proteins, polysaccharides, and lipoproteinpolysaccarides. One speculation is that each of these antigens may have sensitized "uncommitted" lymphocytes to a specific response, thus removing them from the general pool. As such, *in vivo* stimulation by certain antigens might have a cumulatively reductive effect on the availability of PHA-responsive leukocytes in the peripheral blood. Unfortunately, it is difficult to prove the credibility of this speculation because specific antigenic stimulation causes a small *in vitro* response as compared with PHA stimulation.

Another speculation is that perhaps heat-killed *M. tuberculosis* suspended in vaseline and paraffin oil mixture (injected subcutaneously) acted as an amyeloidogenic agent, thus depressing the PHA responsiveness of leukocytes *in vitro*. The basis for this concept is obtained from the knowledge that complete Freund's adjuvant, injected intraperitoneally into mice, induced amyeloidosis and caused a depression of PHA responsiveness of peripheral leukocytes (58). Mice afflicted with amyeloidosis demonstrated lymphopenia, cellular proliferation of the reticuloendothelial system, and functional deficiencies of thymus-dependent immune system (58).

On two occasions, skin testing of tuberculin-sensitized guinea pigs with PPD or OT produced a decrease in percentage of PPD-induced

transformed leukocytes. One speculation to account for this phenomenon is that the skin testing attracted sensitized lymphocytes from a peripheral blood system to skin areas and, as a result, the blood sample did not contain many PPD-sensitized lymphocytes. A second speculation is that the antigen destroyed the sensitized lymphocytes *in vivo*. Both of these possibilities do not seem correct because of the time factor, six days, at which time the system should have recovered from the effect of skin testing. Furthermore, skin testing did not cause an *in vivo* transformation of leukocytes since the percentage of blastoid cells at zero time of culture (six days after skin testing) was less than 2% and was comparable to the number of blastoid cells found in the peripheral blood before the skin tests were applied.

The cause of this *in vitro* "anergy" to a specific antigen is unknown. Perhaps a state of tolerance exists and the PPD-sensitized cells are present but overloaded due to the antigen received *in vivo* via skin testing and the 0.5 µg PPD received *in vitro*. A smaller dose of antigen may have been effective. This might be an explanation for the greater blastogenesis in 6-day non-PPD stimulated cultures obtained on day 35 and day 63, in which transformation values of 29% and 24% were recorded. In these cases, addition of 0.5 ug PPD-inhibited transformation of these leukocytes as indicated by 21.7% and 22.3%, respectively.

Such reasoning indicates that this "anergy" may be due to immune paralysis or immunological unresponsiveness. This reasoning differs from that offered by Sabesin (59) who observed a high degree

of transformation in nonstimulated peripheral blood leukocytes from normal guinea pigs. He reported a 38 to 75% transformation of leukocytes on the fifth day of culture period and that the high degree of transformation was due to the presence of large mononuclear cells which were morphologically indistinguishable from transformed cells. Consequently, he stressed the belief that stimulation and transformation was due to nonspecific factors. This is not compatible with the data presented in Table 6, which shows a decline in non-PPD stimulated transformation as a function of time after immunization and skin testing.

This study has established the guinea pig as a useful animal model for investigating the expression of immunological competency in virus-infected lymphocytes. Moreover, results of this study indicate that prior *in vivo* immunization and skin testing do affect *in vitro* responsiveness of leukocytes to specific antigens and general mitogens.

SUMMARY

The use of guinea pigs as an animal model to study the effect of virus-induced unresponsiveness in peripheral blood leukocytes was established.

Optimal concentration of tuberculin-purified protein derivative (PPD) and culture period needed to get maximum net transformation (12.2%) of leukocytes from Mycobacterium-sensitized guinea pigs were 0.5 µg and six days, respectively. No differences were observed in transformation values of leukocytes to Old Tuberculin stimulation as a function of culture period.

Live Newcastle disease virus (NDV) at an optimal concentration of 1.28×10^{-1} hemagglutination units per 0.1 ml inhibited 97 to 100% of PPD-induced blastogenesis of leukocytes without causing cell death. Ultraviolet irradiation impaired partially the ability of the virus to inhibit specific stimulation of leukocytes.

Furthermore, results indicate that prior *in vivo* sensitization and skin testing do affect *in vitro* immunological responsiveness of leukocytes to specific antigens and general mitogens. Possible explanations for this unresponsiveness are discussed.

The present study has shown the guinea pig to be a useful animal model to study in order to determine the immunological competency of virus-infected lymphocytes.

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