THE DEVELOPMENT OF HYPERSENSITIVE LYMPHOCYTES
IN CELL CULTURE*

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There are several well-known ways to establish cellular (delayed) hypersensitivity
to an antigen. These are (a) by infection with a pathogen such as mycobacteria or
vaccinia virus, (b) by injecting a protein antigen emulsified in complete Freund's
adjuvant, (c) by inoculating histogenetically incompatible cells, and (d) by contact
sensitivity (1-4). Exposure of lymphocytes derived from hypersensitive individuals
to a specific antigen provokes lymphocytes to transform into a blast-like cell type (5,
6), and to release factors that (a) inhibit migration of macrophages (7), (b) are cyto-
toxic in vitro (8), (c) produce an inflammatory reaction if administered into the skin
(9), and (d) have a chemotactic and lymphocyte-transforming effect (10, 11). Despite
recent extensive studies of these so called "lymphokines" (11), little is known about
the induction and properties of the "sensitized lymphocytes" themselves. An in vitro
approach could be expected to clarify at least some aspects of this problem. Therefore,
after our studies on the induction in cell culture of cellular immunity to mouse trans-
plantation antigens (12, 13), we carried out experiments with phytohemagglutinin
(PHA) 1 (14) and pokeweed mitogen (PWM) (15). When PWM-induced blast cells
were plated on embryo fibroblast monolayer that had been conjugated with the
mitogen, the blast cells remained large and lysed the target fibroblasts. The lysis, as
well as the whole culture, appeared identical to graft reaction cultures produced by
exposing rat lymphocytes to a xenogeneic fibroblast monolayer (12, 15). When PWM-
induced blast cells or PHA-induced blast cells were plated on fibroblast monolayers in
the absence of mitogen, the results were entirely different. The blast cells did not lyse
the target fibroblasts; instead, the entire blast cell population transformed to lympho-
cytes (14). The culture system is shown schematically in Fig. 1.

In the present paper we analyze the responsive capabilities of the in vitro
differentiated lymphocytes. It will be shown that the stimulating action of

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Abbreviations used in this paper: ConA, concanavalin A; LA, 5% lactalbumin hydrolysate
in Earle's solution + 5% calf serum; LPC, large pyroninophilic cells; PBS, phosphate-buffered
saline; PHA, phytohemagglutinin; PWM, pokeweed mitogen.
PWM is immunologically specific. In contrast, the other plant lectins which were investigated, PHA and concanavalin A (ConA), did not display any specificity in their stimulating action. It is concluded from an analysis of the experiments that the lymphocytes which differentiate in vitro after stimulation with PWM represent the hypersensitive lymphocytes in cellular immunity.

**Materials and Methods**

*Designations.*—The cell types obtained in our cultures are designated as follows.

**Blast cells:** The large, thymidine-incorporating lymphoid cells formed as a result of trans-

![Diagram of culture system](image)

Fig. 1. Schematic representation of the culture system developed by us (14, 15) and employed in the present study for obtaining blast cells and secondary lymphocytes (See Materials and Methods.)

formation of small lymphocytes. The size distribution (determined with a Coulter counter [Coulter Electronics, Industrial Div., Hialeah, Fla.]) of populations of blast cells shows a peak at a cell volume of 600 $\mu^3$ (14). The enlargement of the lymphocytes is a reversible process that depends on continuous association with the stimulant. Thus, differences in the appearance of cells may reflect differences in the degree of association. The blast cells formed by stimulating lymphocytes with PWM are called "PWM-blast cells," those formed by PHA, "PHA-blast cells," and by ConA, "ConA-blast cells." The blast cells were found to be morphologically identical, whether formed by a mitogen or formed after sensitization of rat lymphocytes on mouse monolayers.

Blast cells are the "killers" in the lytic interaction with target cells. Following Gowans (16) these cells were initially called "large pyroninophilic cells" (LPC). The term "lymphocyte" has also been suggested (17). We prefer to designate the enlarged form simply as "blast cell."
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Primary lymphocytes: Lymphocytes obtained directly from the animals are termed "primary lymphocytes." The commonest cell type in such a suspension is the typical small lymphocyte. The Coulter counter size-distribution plot of a suspension of primary lymphocytes shows a peak at a cell volume of 150 μm³ (14).

Secondary lymphocytes: Lymphocytes which differentiate in vitro from blast cells are termed "secondary lymphocytes." Size distributions of these cells show a peak at 200 μm³ (14). Unlike typical small lymphocytes these cells have a distinct nucleolus. Secondary lymphocytes formed from blast cells which were produced after stimulation with PWM are called "PWM-lymphocytes"; those formed after stimulation with PHA or ConA are termed "PHA-lymphocytes" and "ConA-lymphocytes," respectively.

Animals.—Primary lymphocytes were obtained from lymph nodes collected from rats of the Lewis inbred strain. Both males and females weighing 150–300 g each were used. Embryos for preparation of fibroblast monolayers were obtained from random-bred Wistar rats, Lewis rats, and from mice of strain C3H/FeJ.

Mitogens.—A stock solution of pokeweed (Grand Island Biological Co., Grand Island, N.Y., Lot No. 10033N), containing 1.0 mg dry weight/ml distilled water was made and kept frozen in small tubes. PHA-β (Difco Laboratories, Detroit, Mich., Lot No. 550408) was dissolved in distilled water. A solution containing 20 mg/ml was kept for 3 wk at 4°C. ConA (Miles-Yeda Ltd., Rehovot, Israel, Lot No. 16) was dissolved in a saturated sodium chloride solution. The stock solution, containing 25 mg/ml, was kept at room temperature.

Tissue Culture.—

Embryo fibroblast monolayers: Monolayers from embryos were prepared and maintained as previously described (18, 19). Monolayers used for maintenance of growth and differentiation of blast cells and secondary lymphocytes were prepared by plating 5 × 10⁶ fibroblasts in 10 ml medium (5% lactalbumin hydrolysate in Earle’s solution + 5% calf serum [LA]) onto 100 mm Falcon plastic Petri dishes (Falcon Plastics, Los Angeles, Calif.). In some cases 1 × 10⁶–2 × 10⁶ fibroblasts were plated onto 60 mm dishes. 2–7 days later lymphoid cells were plated on monolayers. Monolayers used for titration of target cell lysis by the 51Cr assay were prepared by plating 0.8 × 10⁶ fibroblasts in 1.5 ml of LA medium, onto 35 mm plastic Petri dishes (see below).

Stimulation of primary lymphocytes with mitogen: Suspensions of lymphocytes were prepared from lymph node cells as described previously (20). The lymph nodes were placed in phosphate-buffered saline (PBS) and forced through wire mesh using a syringe plunger. The resulting suspensions were resuspended in Dulbecco’s medium + 15% horse serum. The horse serum (Grand Island Biological Co.) was inactivated by incubation at 56°C for 30 min. Approximately 95% of the cells in the suspensions were typical small lymphocytes. Mitogens were added to the suspensions to give the following concentrations: PWM, 0.005 ml of stock solution/ml of medium (5 μg/ml); PHA, 0.003 ml of stock solution/ml of medium (60 μg/ml); and ConA, 0.001 ml of stock solution/ml of medium (25 μg/ml). 3 × 10⁶ cells in 4 ml were then plated onto 60 mm Falcon Petri dishes. The cultures were incubated at 37°C in a humidified incubator with a flow of 7% CO₂ in air. Cultures with PHA or ConA were incubated for 3 days while cultures with PWM were incubated for 4 days.

Growth of blast cells on embryo monolayers: After 3–4 days of incubation of primary lymphocytes with a mitogen, the cultures were composed predominantly of blast cells. The blast cells were harvested, washed once with medium, and resuspended in a fresh, mitogen-free culture medium. The cells were counted in a hemocytometer. Small cells, mostly typical lymphocytes, and large cells, mostly blast cells, were counted separately. The two cell types stand in sharp contrast and are easily distinguished in these cultures. 10 ml of the cell suspension were plated onto a Wistar rat embryo monolayer, in a 100 mm Petri dish, at a concentration of 2 × 10⁶ cells/dish. In order to obtain suspensions of secondary lymphocytes the cultures were further
incubated for at least 3 days. The medium was changed every 4th day by decanting 7 ml from the medium surface and adding 8 ml of fresh medium.

Cell transformation: Cell transformation was assessed by measuring the uptake of thymidine-3H as described by Evans and Norman (21). The lymphoid cells, cultured on embryo monolayers, were harvested by gentle pipetting. Cell number was determined and the suspension was divided into three equal parts. Each part was centrifuged and resuspended in 1 ml of Dulbecco's medium. One μCi of thymidine-3H (methyl-T, Radiochemical Centre, Amersham, England, specific activity 5 Ci/mi) in 0.1 ml PBS was added to each tube. The mixture was incubated for 2 hr at 37°C. Triplicate samples of 0.1 ml from each tube were then placed on 2.5 cm GF/c Whatman fiberglass papers. The papers were washed once with saline, twice with 5% trichloroacetic acid, twice with absolute ethanol, and then dried completely by warming under an infrared lamp. The papers were then placed in vials containing 10 ml of scintillation solution (4 g of 2, 5-diphenyloxazole [PPO] and 0.05 g 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]benzene [POPOP] per liter of toluene), and counted in a model 3320 Packard Tri-Carb scintillation counter (Packard Instrument Co., Downers Grove, Ill.). Thymidine-3H uptake is expressed as counts per minute in a sample of 0.1 ml of suspension. Background counts were subtracted from each sample. The standard error for triplicate samples rarely reached 15%. Since it was found unnecessary to calculate whether the differences obtained are significant, the standard error was not included in the results.

Target cell lysis: The degree of target cell lysis was assayed by the release of 51Cr. The assay method has previously been described in detail (19). Briefly, 0.8 X 10^6 Wistar or C3H fibroblasts in 1.5 ml of LA medium were plated in 35 mm Falcon plastic Petri dishes. 24 hr later the cultures were X-irradiated with 2000 R (19). The medium was then replaced with 1 ml of LA medium containing 1.5 μCi of sodium chromate-51Cr (Radiochemical Centre, Amersham, England). After 24 hr the medium was again replaced with 1.5 ml of fresh medium and the plates were incubated for an additional 2-4 days. For titration, twofold serial dilutions of a suspension of lymphoid cells in 1.5 ml of Dulbecco's medium with 15% horse serum were plated onto the 51Cr-labeled monolayers. Since the standard deviation in replicate plates did not exceed 2% (22), only duplicate plates were prepared for each dilution. The cultures were incubated at 37°C for the desired period and processed as described previously (19). The medium from each plate was collected and 1.5 ml of PBS were added to the monolayer. The plates were then shaken and the PBS was collected and combined with the medium. This represents the “medium fraction.” The monolayers remaining in the plates were overlaid with 1.5 ml of trypsin solution and the plates were incubated for 20 min. After shaking, the trypsinized suspension was removed, 1.5 ml of distilled water was added to the plates, and 30 min later the supernatant was combined with the trypsin suspension. This fraction represents the “fibroblast fraction.” Each fraction, containing 3 ml of fluid, was counted in a well-type iodide sodium Crystal Packard Auto-Gamma Spectrometer (Packard Instrument Co.). The total activity in a plate is given by the radioactivity obtained in the fibroblast fraction plus the radioactivity in the medium fraction. Lysis is expressed as the per cent of the total radioactivity remaining in the fibroblasts, e.g.,

\[
\frac{\text{Radioactivity in fibroblast fraction}}{\text{Radioactivity in fibroblast fraction + radioactivity in medium fraction}} \times 100
\]

RESULTS

Formation of Blast Cell Populations after Stimulation of Primary Lymphocytes with Mitogens.—Lymphocyte transformation has been studied extensively and only a brief account will be required here. We used four transforming agents to
stimulate rat lymphocytes. These are xenogeneic (mouse) or allogeneic fibroblast monolayers, PWM, PHA, and ConA. Observations made by us have indicated that our cultures can be divided into two main groups. The first group includes PHA and ConA cultures, and the second, xenogeneic monolayers and PWM. The first group manifests the following characteristics: With the proper mitogen concentration total cell agglutination is obtained. The cells adhere tightly to each other forming large clumps soon after plating. The entire population in the clump is transformed; hence, essentially all the lymphocytes in the culture become blast cells. The second group is characterized by the following features: The cells are not agglutinated; with PWM only a few loosely arranged clumps appear 2 days after plating of the lymphocytes. Unlike the first group, it is not possible to obtain total cell agglutination with these cultures by increasing the mitogen concentration. During the 2nd or 3rd day a very small proportion of the cells are seen as transformed cells while

### Table I

| Mitogen | No. of days in culture | No. of blast cells* per plate (X10⁶) | Blast cells in the culture |
|---------|------------------------|-------------------------------------|---------------------------|
| PHA     | 3                      | 7.6                                 | 79.5                      |
| ConA    | 3                      | 12.7                                | 84.5                      |
| PWM     | 4                      | 10.1                                | 83.3                      |

* 30 X 10⁶ rat lymphocytes were grown in 4 ml of medium in 60 mm Petri dishes.

most of the lymphocytes remain unchanged; however, by the 4th and 5th day the PWM-stimulated cultures are composed predominantly of blast cells. This is because of an exponential increase of blast cells by means of mitosis (5–6% of the cells were in mitosis during the 4th and 5th day) and pronounced degeneration of the remaining small lymphocytes (manifested by an increase in the number of small pyknotic bodies). Table I describes the cell composition of mitogen-stimulated cultures of lymphocytes at the time of cell harvest. Although we experienced a certain variability in blast cell development, many cultures showed very high ratios of blast cells.

A similar pattern has been demonstrated in cultures of lymphocytes exposed to xenogeneic monolayers. In this case not more than 3% of the lymphocytes transform, while by the 5th–7th day a pure culture of blast cells was obtained (17). The similar evolution of blast cells in PWM and xenogeneic cultures suggests that the same rate of lymphocyte transformation occurs in both types of cultures.

**Differentiation of Blast Cells into Secondary Lymphocytes**.—When the har-
vested blast cells were plated on a fibroblast monolayer, entirely different cultures were obtained depending on the presence or absence of mitogen. In the presence of PWM, PWM-stimulated blast cells will effect lysis of the target fibroblasts (15). In the absence of PWM the blast cells differentiate into secondary lymphocytes.

Fig. 2 depicts the kinetics of thymidine uptake in a PHA-stimulated culture before and after the cells were plated on monolayers. Blast cells were washed free of PHA and plated on Wistar monolayers on the 3rd day of culture, when PHA-blast cells were the predominant cell type. The next day the culture was still composed entirely of blast cells which continued to take up thymidine at a high rate, but one day later (day 5) the entire population changed to cells smaller in size. This was accompanied by a marked reduction in thymidine uptake. A similar study employing a Coulter counter has also indicated that the entire blast cell population is transformed to small cells under these conditions (14).

The transformation into secondary lymphocytes was particularly clear when PWM-blast cells were plated in the presence and absence of PWM. The rate of incorporation of thymidine-3H into the lymphoid cells is shown in
Table II. A clear difference in cell population composition was seen as early as 21 hr after plating. In the presence of PWM, blast cells remained large, continued to multiply, lysed the monolayer completely, and later disintegrated (15). In contrast, in the absence of PWM the blast cells became conspicuously smaller, could not lyse the monolayer, and transformed to secondary lymphocytes.

The secondary lymphocytes have been characterized in a previous study (14). They resemble small lymphocytes in fixed and stained cultures, in Giemsa smears, and in their motility and general appearance in living cultures. Secondary lymphocytes show a narrow rim of blue cytoplasm and a compact nucleus. Two features place them into a distinct cell category: the presence of a distinct nucleolus and their cell size. As calculated from the volume, typical small lymphocytes have an average diameter of 6.5 μ, and secondary lymphocytes a diameter of 7.3 μ. The average diameter of blast cells was found to be 10.5 μ.

Growth of secondary lymphocytes derived from any of the three mitogens on mouse monolayers indicated that these cells, unlike primary lymphocytes, were not sensitized by the monolayers to produce blast cells capable of target cell lysis. Of particular interest was the finding that secondary lymphocytes exhibited a tendency to concentrate at various sites on the monolayer and to form numerous lymphoid aggregates or patches. This type of culture has been reported previously for primary lymphocytes grown on rat syngeneic monolayers (23). It is interesting to note that in the patches formed by the secondary lymphocytes, blast cells appeared, increased in number because of mitosis, and within a week or two the entire culture reverted back to secondary lymphocytes.

**Addition of Mitogens to Cultures of Secondary Lymphocytes.**—In order to

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**Table II**

| Time after plating | Counts per min |
|-------------------|----------------|
|                   | With PWM | Without PWM |
| hr                |          |             |
| 0                 | 7,500    | 8,500       |
| 21                | 15,000‡  | 7,600       |
| 46                | 5,000    | 80          |

* 1 × 10⁶ PWM-blast cells collected from a 4-day-old culture (approximately 90% blast cells) were plated on C3H monolayers that were prepared 6 days earlier by plating 0.5 × 10⁶ fibroblasts in LA medium in 60 mm Petri dishes.
† Concentration of PWM: 0.01 ml (10 μg)/1 ml medium.
‡ At 21 hr all fibroblasts were lysed.
study the responsive potential of secondary lymphocytes, mitogens were added
to cultures of secondary lymphocytes maintained on Wistar fibroblast mono-
layers. The monolayers in these cultures were rather heavily populated (15 × 10⁶-25 × 10⁶ fibroblasts/100 mm plate), and most of the lymphocytes floated freely in the medium. Addition of mitogen to these cultures was performed as follows. Half of the medium content of 10 ml was decanted from a plate by suction from the surface. Each plate then received 7 ml fresh medium containing a mitogen in the following concentrations per ml of medium: PHA, 0.002 ml (40 µg); PWM, 0.01 ml (10 µg); and ConA, 25 µg. At various times the freely floating cells were harvested by gentle pipetting and the number of cells and rate of thymidine-³H uptake was determined.

Two main manifestations were observed after mitogens were added. The
first was cell transformation, followed by development of toxic conditions killing
every cell. The second was total transformation to blast cells, without develop-
ment of toxicity, followed by a reversion back to lymphocytes. The effects
obtained with PWM were of particular interest. A clear difference was obtained
when this mitogen was added to cultures of PWM-lymphocytes on one hand
and to PHA- or ConA-lymphocytes on the other. When PWM was added to
PWM-lymphocytes total cell transformation could be clearly seen during the
first 24 hr, but toxic conditions soon prevailed killing the whole culture within
48 hr (Fig. 3 and Table III). All lymphoid cells died early and the entire mono-
layer became necrotic. Toxic conditions did not develop when PWM was added
to ConA- or to PHA-lymphocytes (Tables IV and V, Figs. 4-6). Surprisingly,
the addition of PWM to PHA- or ConA-lymphocytes induced considerable
transformation into blast cells but the entire culture survived.

Total or partial toxicity developed also in some cases when ConA was added
to secondary lymphocytes (Table III). ConA was found to be the most powerful
transforming agent and produced total transformation in all three classes of

| Table III | Addition of Mitogens to a Culture of PWM-Lymphocytes |
|-----------|------------------------------------------------------|
| Time after addition of mitogen | PWM | PHA | ConA | No mitogen |
| hr        |     |     |      |          |
| 0         | 3.00| 2.40| 3.42  | N.D.*    |
| 20        | 3.12| 2.40| 3.24  | N.D.*    |
| 60        | 0.60| 6.48| 6.60  | N.D.     |
| 68        | 0†  | 3.26| 0†    | N.D.     |
| 95        | 0   | 3.08| 0     | N.D.     |

* N.D., not determined.
† Necrotic cultures.
lymphocytes. PHA also produced extensive cell transformation in all the secondary lymphocytes. Since toxic conditions did not develop in these cultures, the increase in number of blast cells was followed by a reversion back to secondary lymphocytes (compare Table IV with Fig. 5, and Table V with Fig. 6).

This study indicates that the response of PWM-lymphocytes to PWM is a specific manifestation. On the other hand, the transformation effect of the three mitogens and the toxic effect of ConA do not seem to be specific.

### TABLE IV

**Addition of Mitogens to a Culture of PHA-Lymphocytes**

| Time after addition of mitogen (hr) | No. of cells harvested per plate (X 10⁶) |
|-----------------------------------|----------------------------------------|
| PWM                              | PHA                                    | ConA | No mitogen |
| 0                                | 1.53                                   |      |            |
| 21                               | 1.05                                   | 0.74 | 0.81       |
| 43                               | 2.53                                   | 3.37 | 1.69       | N.D.† |
| 67                               | 5.40                                   | 9.10 | 4.10       | N.D.  |
| 97                               | 4.00                                   | 8.41 | 1.10       | 1.81  |

* Same culture as shown in Fig. 5.
† N.D., not determined.

### TABLE V

**Addition of Mitogens to a Culture of ConA-Lymphocytes**

| Time after addition of mitogen (hr) | No. of cells harvested per plate (X 10⁶) |
|-----------------------------------|----------------------------------------|
| PWM                              | PHA                                    | ConA | No mitogen |
| 0                                | 1.17                                   |      |            |
| 42                               | 1.37                                   | 2.28 | 1.87       | 0.63  |
| 61                               | 2.99                                   | 3.45 | 4.95       | N.D.‡ |
| 86                               | 2.86                                   | 2.64 | 2.94       | 0.33  |
| 108                              | 0.99                                   | 3.57 | 2.21       | N.D.  |

* Same culture as shown in Fig. 6.
‡ N.D., not determined.

**Kinetics of Target Cell Lysis by PWM-Blast Cells and PWM-Lymphocytes:**—We have previously shown that PWM-blast cells lyse target cells in the presence of PWM in exactly the same way as do blast cells (LPC) produced by sensitization of primary lymphocytes on xenogenic monolayers (15). Since secondary lymphocytes transform to blast cells, we studied the kinetics of lysis produced by both PWM-blast cells and PWM-lymphocytes. Blast cells were obtained from 3-day-old cultures of primary lymphocytes grown with PWM. The cells were washed once and divided into two parts. The first part was resuspended in medium containing 0.01 ml of PWM/ml of medium while the second part
was resuspended in medium alone; 0.5 × 10⁶ blast cells were plated onto ⁵¹Cr-labeled target monolayers. At intervals, two plates from each group were assayed for ⁵¹Cr release. The results are shown in Fig. 7. In the group in which cells were plated in the presence of PWM, lysis was clearly evident by 4 hr and proceeded in an accelerated fashion during 28 hr. From 28 hr on the culture conditions deteriorated quickly and the lymphoid cell population died. This is reflected in the slower rate of ⁵¹Cr release. The disintegration of lymphoid cells was followed by death of the surviving fibroblasts in the monolayer (as described in the previous section) since the medium from such cultures became toxic. This type of cell death was found to be morphologically different from lysis, and was termed "necrotic death."

![Graph showing thymidine-²H uptake](image)

**Fig. 3.** Thymidine-²H uptake after the addition of PHA and PWM to PWM-lymphocytes maintained on Wistar fibroblast monolayers. Experimental conditions are discussed in the text. Toxic conditions developed in the culture with PWM.

The two morphologically different phenomena of cell death are well demonstrated in the kinetics of lysis by PWM-lymphocytes (Fig. 8). In this experiment 3-day-old PWM-blast cells were plated on Wistar monolayers. 3 days later, when the entire population had already differentiated into secondary lymphocytes, the cells were collected and resuspended in medium as described for the previous experiment (Fig. 7); however, here part of the lymphocytes were also resuspended in medium containing 25 µg of ConA/ml. In the presence of PWM, all PWM-lymphocytes adhered to the fibroblast monolayer within 3 hr. There was actually no difference in the time of onset of the lytic course between PWM-blast cells and PWM-lymphocytes. In both cases lysis was observed as early as 4 hr after plating; thus, lysis started even before the secondary lymphocytes had enlarged. At 8 hr, when lysis was widespread, the secondary lymphocytes were already conspicuously larger in size and at 16 hr the entire population of PWM-lymphocytes attained the size of blast cells.
Fig. 4. Thymidine-\(^{3}\)H uptake after the addition of PHA and PWM to PHA-lymphocytes maintained on Wistar fibroblast monolayers. Experimental conditions are discussed in the text.

Fig. 5. Thymidine-\(^{3}\)H uptake after the addition of PHA, PWM, and ConA to PHA-lymphocytes maintained on fibroblast monolayer (same experiment as shown in Table IV).
Fig. 6. Thymidine-$^3$H uptake after addition of PHA, PWM, and ConA to ConA-lymphocytes (same experiment as shown in Table V).

Fig. 7. Extent of lysis of Wistar fibroblast monolayers by PWM-blast cells as a function of time.
Fig. 8 reveals an interesting difference in the kinetics of $^{51}$Cr release in cultures containing $2 \times 10^5$ PWM-lymphocytes as compared to cultures containing only $2 \times 10^4$ lymphocytes. At the higher lymphocyte concentration, the release of lymphotoxins (15, 24) killed the blast cells during the period between 24 and 48 hr. This is reflected in a much slower rate of $^{51}$Cr release during this interval. By 48 hr no lymphoid cells survived; however, the toxic medium also killed those fibroblasts that survived the lytic interaction. This is reflected in the increased slope obtained between 48 and 66 hr. Observations at 66 hr

![Graph showing the extent of lysis of Wistar fibroblast monolayers by PWM-lymphocytes as a function of time.](image)

indicated that all the fibroblasts died and the whole culture became necrotic. The slow rate of $^{51}$Cr release obtained during the 66–114 hr period was because of fragmentation and dispersal of the dead cells. An entirely different pattern was obtained when only $2 \times 10^5$ PWM-lymphocytes were plated in the presence of PWM or ConA. At this low concentration toxicity did not develop. The transformed blast cells were widely scattered; they survived during the 5 day incubation period and steadily lysed the target cells. Fibroblasts that escaped the lytic interaction remained alive since necrotic conditions did not develop. This study shows also that a certain degree of fibroblast lysis was brought about by PWM-lymphocytes in the presence of ConA. Here also, total transformation to blast cells occurred.

**Target Cell Lysis by Blast Cells and by Secondary Lymphocytes in the Presence of Mitogens.**—The ability of the three classes of blast cells and secondary
lymphocytes to lyse Wistar target monolayers in the presence of one of the mitogens was tested. In the first experiment (Fig. 9) PWM-, ConA-, and PHA-blast cells were harvested and titrated in the presence of 0.01 ml (10 μg) of PWM/ml of medium. $2 \times 10^6$ PWM-blast cells completely lysed the target monolayer within 21 hr. In contrast, ConA- or PHA-blast cells did not lyse the fibroblasts in the presence of PWM, nor did PWM-blast cells lyse the target cells in the absence of the mitogen. Under these conditions, the entire monolayer survived. A different pattern of target cell lysis was obtained when the blast cells were titrated in the presence of ConA (25 μg/ml) (Fig. 10). PWM- and ConA-blast cells lysed to the same extent. PHA-blast cells were somewhat less effective. These cells looked smaller and more heterogeneous in size, as compared with those incubated in the presence of ConA and PWM. The weaker lysis may then be because of the lesser maturity of the PHA-blast cells. This study indicates that the lysis of target cells by blast cells is specific in the presence of PWM but not specific in the presence of ConA.

The second series of titrations was performed with secondary lymphocytes. The assay in the presence of PWM is shown in Fig. 11. The results of this experiment indicate that specificity in lysis resides in PWM (compare with Fig. 9). Figs. 12 and 13 show titrations in the presence of ConA and PHA, respectively. No lytic specificity is demonstrated by these mitogens. In the presence of PHA and ConA all three classes of lymphocytes released $^{51}$Cr to the same extent.

An analysis of these two series of experiments reveals two phenomena: (a) There is no direct correlation between transformation of secondary lymphocytes and target-cell lysis. PWM also transforms ConA- and PHA-lymphocytes; however, the resulting blast cells do not lyse fibroblasts. (b) Lysis is more efficient at lower concentrations of secondary lymphocytes, slowing down gradually as cell concentration increases without ever reaching complete lysis. (Compare end points of curves in Fig. 11 with complete lysis obtained with blast cells in Fig. 9.) As shown in Fig. 11, at the lowest concentration of lymphocytes lysis was over 20 times more efficient with PWM-lymphocytes than with ConA- or PHA-lymphocytes. Approximately $6 \times 10^6$ ConA-lymphocytes or $1 \times 10^6$ PHA-lymphocytes were required to produce the same rate of $^{51}$Cr release as obtained by $3 \times 10^4$ PWM-lymphocytes. Observation of the cultures has clearly indicated that the survival of transformed secondary lymphocytes was much greater at lower concentrations than at higher concentrations. In the latter case transformation of secondary lymphocytes was followed by degeneration due to the development of toxicity in the medium.

Since PWM-lymphocytes transform totally within 16 hr, no conspicuous difference was obtained in the pattern of lysis when blast cells and secondary lymphocytes were titrated for lysis on Wistar monolayers in the presence of a mitogen; however, exposure of PWM-lymphocytes to PWM produced stronger toxic conditions that led to culture necrosis.
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Fig. 9. Titration of the lytic reaction of three classes of blast cells plated on Wistar fibroblast monolayers in the presence of PWM.

Fig. 10. Titration of the lytic reaction of three classes of blast cells plated on Wistar fibroblast monolayers in the presence of ConA.
DISCUSSION

Schwartz and Rieke have shown that PHA-induced blast cells produce lymphocytes after in vivo inoculation (25, 26). The present study describes this differentiation in an in vitro system. Using this simplified system several fundamental properties of lymphocytes have been revealed. Thus, it has been seen that: (a) lymphocyte transformation into blast cells requires continuous association with the stimulant, (b) blast cells will remain large as long as the stimulant is present, (c) when a population of blast cells is dissociated from

![Graph showing the lytic reaction of three classes of secondary lymphocytes plated on Wistar fibroblast monolayers in the presence of PWM.](image)

Fig. 11. Titration of the lytic reaction of three classes of secondary lymphocytes plated on Wistar fibroblast monolayers in the presence of PWM.

the stimulant the entire population transforms to lymphocytes, and (d) reinoculation of the stimulant results in reenlargement of the lymphocytes.

The experiments described here reveal that the stimulating action of PWM on lymphocytes is immunologically specific. PHA and ConA on the other hand, display no specificity in their stimulating effect on lymphocytes. When primary lymphocytes are exposed to PWM, only a small fraction of the lymphocytes transform into blast cells. In this respect the situation is similar to the stimulating effect of xenogeneic or allogeneic fibroblast monolayers on primary lymphocytes, which is also immunologically specific (12). In the latter case the fraction of lymphocytes transformed was found to be no more than 3% (27).
Observations of the PWM-induced and monolayer-induced cultures (as well as ConA cultures) indicate that lymphocyte transformation in PWM cultures is of the same magnitude. Such cultures differ markedly from PHA and ConA cultures where all cells are clumped and transformation seems to be total. It is of interest to note that the kinetics of thymidine incorporation in PWM cultures was found to be different from that in PHA and ConA cultures (28).

The pattern of blast cell development was found to be identical to that shown by monolayer-induced cultures (17, 27). In the continuous presence of PWM the blast cells increase exponentially by mitosis during the 3rd–7th day, whereas the rest of the lymphocytes die. As a consequence, pure populations of PWM-blast cells are obtained. If these blast cells are exposed to target cells in the presence of PWM they immediately adhere, and lysis of target cells follows. Lysis is specific, since ConA- and PHA-blast cells are unable to lyse target cells in the presence of PWM, and PWM-blast cells are unable to lyse target cells in the absence of PWM.

The specific activation of the lymphocytes by PWM is thought to occur along the following lines: PWM, a glycoprotein plant lectin with a molecular weight of 32,000 (29, 30), combines with cells, most probably with the cell membrane. After this combination, certain lymphocytes are able to recognize the PWM as a "transplantation antigen" and are activated, as in a graft reaction, to produce effector cells which lyse target cells. The specific determinants reside in the PWM. In the absence of the mitogen the blast cells condense and transform to long-lived lymphocytes. Since the latter can be reactivated by PWM to undergo blastogenesis and evoke specific lysis, it appears that such lymphocytes represent the memory cells that bear the specificity to the PWM antigenic determinants.

Support for the foregoing interpretation comes from in vivo studies with PWM. As in an allograft reaction, cellular hypersensitivity to PWM was produced in guinea pigs by simple injection of PWM without the need of emulsification in Freund's adjuvant. The sensitized animals produced much stronger skin reaction (analogous to second-set rejection) than the unsensitized animals (unpublished data).

Lytic cultures were also obtained with ConA. The lytic interaction was found to be identical to that obtained by monolayer-induced and by PWM-induced cultures. In contrast, however, ConA displayed no specificity in lysis. All three classes of blast cells or secondary lymphocytes lysed target cells to a certain degree in the presence of ConA.

PHA presents a different situation; PHA agglutinates and transforms the lymphocytes. At increased concentrations this mitogen becomes readily toxic. In cultures where massive transformation occurs in the presence of a fibroblast monolayer, release of lymphotoxins causes deterioration of the culture (14, 15, 24). In the present study a nontoxic concentration of PHA was used; however,
despite total cell transformation, definite lysis was not obtained. Therefore, the question of whether or not a lytic interaction between lymphocytes and fibroblasts occurs in the presence of PHA remains open.

It is important to consider the problem of secondary lymphocyte transformation by plant lectins independently of the lytic interaction, since the data indicate that there is no necessary correlation between the two. Transformation may provide effector cells which directly lyse target cells. This is clearly manifested when PWM-lymphocytes are exposed to PWM-conjugated target cells. At low concentrations of lymphocytes (to avoid toxification of the culture medium) the PWM-specific adherence of the PWM-lymphocytes to target cells is soon followed by simultaneous lysis and transformation (Hollander and Ginsburg, to be published). By 16 hr, the entire population transforms into blast cells. The enlarged cells continue to lyse target cells over a period of at least 4 days.

A second type of transformation, or blastogenesis of secondary lymphocytes, takes place on fibroblast monolayers without the occurrence of a lytic interaction. This blastogenesis is also obtained “spontaneously” in the absence of mitogen when secondary lymphocytes are plated on fibroblast monolayers. The lymphocytes aggregate and form lymphocyte patches, as was described previously in cultures of primary lymphocytes (23). Blast cells appear in large number in these patches. A similar kind of blastogenesis is obtained when PHA is added to cultures of secondary lymphocytes or when PWM is added to ConA- and PHA-lymphocytes. In all these cultures, the blast cells revert back to secondary lymphocytes. Usually this reversion results in an increase in the number of secondary lymphocytes.

A series of experiments have been designed to test whether blast cells and secondary lymphocytes, formed after exposure of PWM-lymphocytes to PHA, retain their specific ability for cell lysis. The results (Hollander and Ginsburg, to be published) indicate that the memory specific to PWM is virtually retained after stimulation of PWM-lymphocytes by PHA. Thus, in this study nonlytic blastogenesis resulted in the replication of hypersensitive lymphocytes.

**SUMMARY**

An in vitro cell-mediated immune response to pokeweed mitogen (PWM) is described. Rat lymphocytes were stimulated by PWM, by phytohemagglutinin (PHA), and by concanavalin A (ConA). In the presence of PWM only a fraction of the lymphocytes underwent blastogenesis. This was in contrast to the apparent total blastogenesis obtained in response to PHA or ConA. When blast cells derived from each of the mitogens were plated on rat fibroblast monolayer in the absence of mitogen they differentiated into a distinct type of lymphocyte termed “secondary lymphocyte.” Addition of mitogens to cultures of these lymphocytes resulted in a retransformation to blast cells.

The secondary lymphocytes were tested for their ability to effect lysis in the
presence of each of the three mitogens. In the presence of PWM, lysis of fibroblasts produced by PWM-lymphocytes was considerably more efficient than lysis obtained by ConA- or PHA-lymphocytes. No difference in effect on target fibroblasts was obtained when the three types of secondary lymphocytes were tested in the presence of either PHA or ConA. The stimulating action of PWM on lymphocytes was shown to be immunologically specific. No such specificity was found in the case of PHA or ConA.

The results are interpreted to indicate that PWM combines with cell membranes and acts on the lymphocytes as a “transplantation antigen.” Lymphocytes capable of responding to “PWM-transplantation antigen” transform to blast cells capable of specifically lysing PWM-conjugated fibroblasts. In the absence of the mitogen, PWM-induced blast cells differentiate to lymphocytes hypersensitive to PWM.

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