QUANTITATIVE STUDIES ON THE MIXED LYMPHOCYTE INTERACTION IN RATS

I. CONDITIONS AND PARAMETERS OF RESPONSE*

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Numerous studies have shown that cultures of leukocytes can be stimulated by a variety of agents—including phytohemagglutinin, pokeweed mitogen, tuberculin antigen, and homologous leukocytes—to undergo transformation to pyroninophilic blast cells, synthesize nucleic acid, and divide (1–6). It is generally conceded that it is the lymphocyte fraction of the leukocyte population which responds to the mitogenic stimuli.

The proliferative response incited by foreign cells (6, 7) or cell products (8) in a “mixed lymphocyte interaction” poses several interesting questions. For example, although there is some evidence that the magnitude of the response is governed by the degree of immunogenetic disparity between the leukocyte donors (3, 7), it is by no means certain that the stimulatory factors are transplantation antigens. Also to be resolved is whether this in vitro mitotic activity of lymphocytes represents a primary immune response at the cellular level.

Many of the studies on the proliferative responses of leukocytes have been conducted with cells of human origin mainly because of the difficulties encountered in establishing cultures with cells procured from laboratory animals. Serious drawbacks associated with the use of human culture material include a limited range of acceptable experimental manipulation as well as a lack of genetic control of the cell donors.

The present report describes the conditions under which reproducible responses can be obtained with mixed cultures of rat peripheral blood leukocytes as well as the parameters of this response is quantitative terms. Evidence will be presented that (a) proliferative responses occur only when the two donors differ with respect to important transplantation antigens determined by alleles at the AgB locus, and (b) the induced mitotic activity of mixed

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cultures represents an immunologic response at the cellular level against histocompatibility antigens.

Materials and Methods

Many of the procedures employed in this study have been described elsewhere (9). However, certain modifications were essential for culturing rat cells.

The animals employed were adult rats of the following inbred strains and their F1 hybrids: Lewis, BN, DA, Fischer, and BH. Most of these strains are known to differ at the important AgB histocompatibility locus (see Table IV).

Preparation of cell suspensions: Blood (8 volumes) was drawn from adult rats by cardiac puncture into disposable syringes containing citrate (Na salt, 1 volume, final concentration 0.25% w/v) and dextran (1 volume, final concentration 1% w/v). Erythrocytes were removed by sedimentation either in the syringe (needle end up) or in 20 × 150 mm Pyrex tubes at room temperature. After removal of the leukocyte-rich plasma fraction, an equal volume of Hanks’ balanced salt solution (HBSS) containing citrate and dextran was added and the erythrocyte fraction allowed to settle again to increase the yield of leukocytes. The leukocyte fractions were pooled, washed twice in chilled HBSS containing heparin (1 IU/ml), and suspended in a convenient volume of culture medium (5-10 ml). An important advantage of collecting lymphocytes from the peripheral blood for culture material is that the same donor can be used repeatedly. 5 or 6 ml of blood can be drawn from a 250-g rat at biweekly intervals with no untoward effects.

Cell suspensions were also prepared from the lymph nodes and spleens by techniques fully described elsewhere (10) and these were washed and suspended in media as above.

Crude differential counts with the aid of a phase contrast microscope were performed on aliquots of cells diluted in 2% acetic acid. Cells were distinguished as “mononuclear”—predominately lymphocytes and hereinafter referred to as such—or as “granulocytes.” By this procedure, it was possible to harvest approximately 7-10 million leukocytes per original milliliter of blood, 70-90% of which were lymphocytes.

Sera employed as a supplement to the culture media (10-20%) were obtained from many sources. Calif° and fetal Calif° rabbit,° and hamster° sera were obtained commercially. Human° (type AB, Rh+) plasma was obtained from a blood bank, and guinea pig and rat sera were prepared according to standard laboratory procedures. The latter was prepared from cardiac blood that was allowed to clot at room temperature for 30 min, then stored at 4°C for 4 hr after which time the serum was removed from the clot. Records of the age, number of times frozen and thawed, and whether or not they had been heat-inactivated were maintained for all of the sera.

Conditions of culture: The cells were cultured in a total volume of 1 ml media in 13 × 100

1 BH designates an inbred stock of black hooded animals obtained from A. Z. Hodson of the Pet Milk Co. These rats originated from a Sprague-Dawley × Long-Evans cross and were brother–sister mated for 22 generations. When received in this department, skin grafts exchanged between them were accepted indefinitely.

2 Falcon Plastics, J. E. Franklin Co., Philadelphia, Pa.

3 Dextran; MW 240,000; Pharmachem Corp., Bethlehem, Pa.

4 Grand Island Biochemical Co., Grand Island, N.Y.

5 Culture medium: 2X Eagle’s BME, 1X Earle’s salts; containing glutamine (Flow Laboratories, Rockville, Md.), penicillin (100 IU/ml), and streptomycin (100 µg/ml).

6 Flow Laboratories, Rockville, Md.

7 Plasmas from Blood Bank, Hospital of University of Pa.
mm disposable flint glass tubes. The tubes were closed with Morton stainless steel caps and maintained upright in a 95% air-5% CO₂ humid atmosphere at 37°C.

The assay procedures employed differ only slightly from those previously reported (9). At appropriate times after initiating the cultures, 0.1 ml of phosphate-buffered saline containing 0.25 μC tritiated thymidine was added. The tubes were returned to the incubator for a subsequent 16 hr culture period at which time they were removed, the cells agitated on a vortex mixer, and washed with 10 volumes of chilled saline. The cell pellets were then broken up with a vortex mixer, resuspended in 1 ml of cold trichloroacetic acid (TCA, 5% w/v) and frozen. If convenient, the samples were stored in this state for further processing. The TCA precipitates were collected onto glass fiber filter pads, with suction filtration, washed with 10 volumes of cold 5% TCA, and dehydrated with acetone. The radioactivity of the samples was assessed in 2 ml of scintillation fluid using liquid scintillation techniques.

Each experiment was conducted in triplicate at least three times and, with this assay system, the counting efficiency was approximately 45%. Counts among replicate cultures rarely varied by more than 15%. Background was approximately 11 cpm and was subtracted automatically from all data.

RESULTS

1. Conditions for Response.—For reasons which are not entirely clear, attempts to induce proliferative responses to phytohemagglutinin or other mitogenic agents in blood leukocytes derived from animal species other than man have met with only limited success. For the studies reported here, it was essential to devise procedures for the successful culture of rat leukocytes on a consistent and reproducible basis. Therefore, as a starting point, it was decided to determine whether culture conditions previously demonstrated to sustain proliferative responses of human lymphocytes could be successfully employed for the stimulation of rodent lymphocytes. The amount of incorporation of radioactive thymidine into TCA precipitable cell fractions, measured with liquid scintillation counting techniques, provided a rapid and sensitive means for assessing the degree of cell proliferation.

Cells harvested from the peripheral blood or spleens of DA rats were committed to 1 ml cultures (10⁴ lymphocytes/culture) in the presence of the mitogen, phytohemagglutinin (PHA; 0.001 ml/ml). The medium was supplemented with serum (15% v/v) derived from various sources. The cultures were terminated after 5 days of incubation and the results, expressed in terms of counts per minute per culture (mean of three cultures), are presented in Tables I and II. While PHA cultures of rat blood lymphocytes or splenic cells in medium supplemented with human plasma (HP), calf (CS),

9 Becto, Vineland, N. J.
10 Specific activity 6.7 c/mmole as methylthymidine-³H; New England Nuclear Corp., 575 Albany St., Boston, Mass.
11 Reeve Angel, grade 934 AH, Arthur H. Thomas, Phila., Pa.
12 Packard Tri-Carb; Model 3365. A scintillation fluid was used consisting of toluene (3.78 liters), PPO 15.1 g, and POPOP 1.14 g.
13 Phytohemagglutinin, P form, Difco Laboratories, Inc., Detroit, Michigan.
TABLE I

Peripheral Blood Leukocytes from DA Rats Cultured with PHA in Media Supplemented with Various Sera

| Sera               | PHA* added | Mean cpm per culture |
|--------------------|------------|----------------------|
| Human §            | +          | 4980                 |
|                    | -          | 43                   |
| Rabbit             | +          | 4                    |
|                    | -          | 4                    |
| Calf               | +          | 5223                 |
|                    | -          | 633                  |
| Fetal calf         | +          | 4902                 |
|                    | -          | 294                  |
| Rat (stored)       | +          | 188                  |
|                    | -          | 16                   |
| Rat (fresh)        | +          | 5179                 |
|                    | -          | 24                   |

* PHA-P (0.001 ml/ml).
† Cultures conducted in triplicate; 1.0 X 10⁶ lymphocytes/ml per culture.
§ Plasma.

TABLE II

Splenic Lymphocytes from DA Rats Cultured with PHA in Media Supplemented with Various Sera

| Sera                          | PHA* added | Mean cpm per culture |
|-------------------------------|------------|----------------------|
| Human §                       | +          | 4875                 |
|                                | -          | 1083                 |
| Rabbit                        | +          | 18                   |
|                                | -          | 7                    |
| Calf                          | +          | 1270                 |
|                                | -          | 490                  |
| Fetal calf                    | +          | 1540                 |
|                                | -          | 732                  |
| Rat (stored) (pooled)         | +          | 30                   |
|                                | -          | 19                   |
| Rat (fresh) (BN strain)       | +          | 5030                 |
|                                | -          | 37                   |

* PHA-P (0.001 ml/ml).
† Cultures conducted in triplicate; 1.0 X 10⁶ lymphocytes/ml per culture.
§ Plasma.

or fetal calf serum (FCS) displayed considerable uptake of thymidine-³H. Significant levels of activity were also seen with control cultures—especially of splenic cells—lacking PHA. HP qualifies as an acceptable medium supplement for cultures of blood leukocytes, but not for splenic cell cultures. With
fresh rat serum (FRS), on the other hand, not only did PHA produce a great amount of proliferation in blood lymphocyte and spleen cell cultures, but background activity in control cultures was minimal.

Peripheral blood lymphocytes from BN rats were cultured \(10^6/\text{ml}\) in 1 ml volumes for different intervals with various threefold dilutions of PHA (starting with 0.03 ml/ml) in media supplemented with HP (10% v/v). The results, plotted in Fig. 1, show that there was a definite dose-dependent response similar to that of human lymphocytes to PHA under similar conditions (9). Experiments with Pokeweed mitogen\(^1\) (PWM) gave comparable results, i.e., peak responses occurred at 3 days of culture at a concentration of PWM slightly higher than PHA (0.003-0.01 ml/ml).

Several experiments were conducted to determine whether or not FCS, HP, and FRS would be helpful in obtaining successful responses with mixed

\(^1\text{Pokeweed mitogen, Grand Island Biochemical Co., Grand Island, N. Y.}\)
cultures of homologous lymphocytes. The results of these experiments are compiled qualitatively in Table III and some of them are plotted in Fig. 2 (Expts. 56, 57, 58, 60, 61, 62). These findings can be summarized as follows: (a) Mixed cultures containing a total of 30 million splenic or blood lymphocytes in a volume of 1 ml showed no response over a 2-7 day period whether in media

| Expt. | Cells cultured* | Media supplement | Response† to PHA |
|-------|-----------------|-----------------|-----------------|
|       | Type            | Source†         | Conc. 2 4 6 7 9 11 |
| 50    | BL 15 + 15      | HP 15           | - - - - +       |
| 55    | SL 15 + 15      | FRS 10          | - - - - +       |
| 56    | SL 1 + 1        | FCS 15          | ? ± - + +       |
| 57    | SL 1 + 1        | FRS 15          | - + + + ±       |
| 58    | SL 1 + 1        | HP 15           | ± + ± ± +       |
| 59    | BL 15 + 15      | FRS 10          | - - - - ±       |
| 60    | BL 1 + 1        | FCS 15          | + + + + ±       |
| 61    | BL 1 + 1        | FRS 15          | - + + + ±       |
| 62    | BL 1 + 1        | HP 15           | - - - = +       |
| 63    | BL 1 + 1        | FRS 15          | ± + + + +       |
| 64    | SL 1 + 1        | FRS 15          | ± + + + - - - - |
| 67    | BL 1 + 1        | FRS 30          | - ± + + + ± - - |

* Cells maintained in cultures with 1 ml media; BL, blood lymphocytes; SL, splenic lymphocytes.
† HP, human plasma; FRS, fresh rat serum; FCS, fetal calf serum.
‡ Day on which response was measured: — indicates no response of any significance over that occurring in control, unmixed cultures; ? indicates response of questionable significance, i.e., counts from control cultures were high; ± indicates positive response, but only slightly above control levels which were not always appropriately low, + indicates highly significant proliferative response, no responses in control cultures.
§ Proliferative response of mixed cells incubated with PHA and measured on 2nd, 3rd, or 4th day. This represents a positive control for viability.

containing HP or FRS—even though the cells could be shown to be viable by their response to PHA (Expts. 50, 55, 59); (b) less crowded cultures containing a total of 2 million mixed homologous blood lymphocytes (1 million + 1 million) responded well in media containing FRS (Expts. 61, 63, 67) but not as well in media containing HP or FCS (Expts. 60, 62); (c) thymidine uptake levels in control, unmixed cultures of blood lymphocytes containing 2 million cells/ml were especially low in media containing FRS (see Fig. 2); (d) mixed
cultures of splenic cells gave good responses in FRS (Expts. 57, 64), and showed little or no response in HP and FCS (Expts. 58, 56; Fig. 2); (c) responses of control splenic cells in the various sera tended to be disturbingly high (Fig. 2); and (f) there was no response in the mixed cultures before the 4th day.

2. Kinetics of Response of Mixed Cultures of Peripheral Blood, Lymph Node, and Splenic Cells.—To determine more accurately when proliferative responses develop in mixed cultures and to compare the proliferative capacity of lymphocytes of different tissue origin the following experiments were conducted. Lymphocytes obtained from the peripheral blood, lymph nodes, and spleens of Lewis and BN rats were mixed and committed to culture under standard conditions, i.e., each culture contained 2 million cells (1 million Lewis and 1 million BN) in 1 ml of medium supplemented with fresh BN serum at a concentration of 10%. These cultures were terminated daily over a period of a week after an overnight exposure to 0.25 μc tritiated thymidine (16 hr). The data (Fig. 3) show that only minimal responses occurred before the 4th day with
lymph node and peripheral blood lymphocytes and the responses were maximal at 7 days. Mixed splenic cells, on the other hand, showed some activity during the 2nd and 3rd days and the response occurring afterwards was complicated by significant incorporation of thymidine into control cultures.

3. Proliferation by Small Lymphocytes.—Much of the available evidence that proliferation in leukocyte cultures involves primarily the small lymphocytes has come from studies in which PHA was used as a mitogen. To determine whether proliferation in mixed cell cultures also involves the small lymphocyte,

![Graph showing proliferation response of mixed cultures of blood, lymph node, or splenic lymphocytes from Lewis and BN donors.](image)

**Fig. 3.** Proliferative response of mixed cultures of blood, lymph node, or splenic lymphocytes from Lewis and BN donors.

cells from the thoracic lymphatic duct (11), predominately small lymphocytes, were used as culture material.

Also, since dextran is used in procedures for removing the bulk of the red cells during the preparation of blood leukocyte suspensions, it seemed important to determine what influence this agent might have on the proliferative activity of cultured cells. To do this, suspensions of lymphocytes were obtained from the thoracic lymphatic duct of DA and BN rats. The concentration of cells in these suspensions was adjusted to 5 million/ml and aliquots of the DA and BN lymphocytes were allowed to stand at room temperature for 30 min with or without dextran (final concentration 1% w/w). Following this, the cells were washed with HBSS as usual and committed to culture both in mixed and singly in control cultures. The results of the experiment (Fig. 4) show an identical re-
sponse on the part of the mixed cultures, whether or not the cells have been previously exposed to dextran. As before, control, unmixed cultures of cells showed no response; for the sake of clarity only the results of control cultures of dextran-treated DA thoracic duct lymphocytes are presented.

These data provide strong evidence that it is the small lymphocytes which are the responding cells in a mixed leukocyte culture. Further evidence that circulating small lymphocytes are the proliferating cells in the mixed lymphocyte interaction stems from the following experiment employing "purified" blood lymphocytes. Leukocyte suspensions were prepared as before by dextran sedimentation from BN and L/BN F1 rat blood and adjusted to a concentration of 2.5 million lymphocytes/ml in growth medium containing 15% FRS. Aliquots of 25 million of these cells were subjected to one or the other of two preincubation procedures—one of which served to delete "contaminating" granulocytes and monocytes, and the other under somewhat similar conditions, but which did not favor the removal of granulocytes.

The first group were preincubated at 37°C for 1.5 hr in plastic T flasks having a surface area of 75 cm² (2). The unattached cells were carefully decanted and the process repeated. The second group were placed in plastic conical centrifuge tubes (2) and maintained in an incubator under similar conditions. Following

![Graph](image.png)

**Fig. 4.** Responses of mixed cultures of thoracic duct lymphocytes; (+D), cells pretreated with dextran; (−D), not treated.
these pretreatments, the cells were spun down, adjusted to an appropriate concentration in terms of lymphocytes per milliliter (regardless of the other cell types present) and committed to culture under the standard conditions of 2 million lymphocytes/culture of 1 ml in medium containing 10% FR. Microscopic examination of the "purified" and "nonpurified" suspensions in acetic acid showed that fewer than 1% of granulocytes were present in the former and approximately 25% in the latter.

The results of this experiment are presented in Fig. 5 and show that equal numbers of lymphocytes in a mixed lymphocyte culture undergo a greater proliferative response in the presence of granulocytes than in the absence of these cells. Again, the response of unmixed cultures of cells was nil and these control values are plotted as a single line for the sake of clarity.

4. Influence of Peritoneal Macrophages on the Mixed Lymphocyte Interaction.— The experiments presented above demonstrate that homologous mixtures of relatively pure suspensions of circulating lymphocytes obtained from the
thoracic lymphatic duct, or partially purified lymphocyte suspensions prepared from the leukocyte fraction of the blood, are capable of responding by proliferation, to homologous cells in culture. The findings presented do not exclude the possibility that the presence of other cell types, particularly macrophages, might have some influence on the magnitude and kinetics of the proliferative response. This question is particularly pertinent since numerous studies have stressed the role of macrophages in the initiation of immune responses in vivo (12).

![Graph](image)

**Fig. 6.** Response of mixed cultures of lymphocytes in the presence or absence of peritoneal exudate ("macrophage") cells.

To provide some information on this point, cells were flushed from the peritoneal cavities of normal, unstimulated BN rats, washed twice in Hank's balanced salt solution containing heparin, and then suspended in medium at a concentration of 1 million cells/ml. 1 ml aliquots of these cells were dispensed into culture tubes, and placed in an incubator overnight. During this period many of the cells—"macrophages"—attached to the bottom of the glass culture tubes, and the unattached cells were decanted and discarded. Immediately afterwards mixtures of BN and BN/DA F₁ blood lymphocytes were added (1 million + 1 million culture) to some of the macrophage tubes. Controls included cultures of mixed cells without macrophages, and unmixed cells with and without macrophages. The results of this experiment (Fig. 6) were clear-cut; there was no substantial difference either in the magnitude or the kinetics of the uptake of tritiated thymidine by mixed suspensions of lymphocytes regardless of the presence or absence of macrophages. Control cultures containing unmixed
BN/DA or BN lymphocytes and macrophages incorporated small, but significant amounts of the radioactive precursor.

5. Reproducibility of the Proliferative Response.—To provide information on the uniformity of the proliferative response by blood lymphocytes from various individuals of the same inbred strain against a common pool of homologous cells, leukocytes were collected and prepared separately from four young adult male DA rats and cultured with pooled leukocyte suspensions prepared from a panel of three BN/DA F1 males. The use of parental and F1 cells in this experiment permits a unilateral response by the parental cells only (see below). The results (Fig. 7) show that uptake of tritiated thymidine by cells of different DA rats (DA1–DA4) after various times in cultures with BN/DA cells is strikingly similar especially during the ascending portion of the response curves.

6. Reactions of Mixed Cultures of Peripheral Blood Lymphocytes Derived from
Various Isogenic Strains of Rats and their F₁ Hybrids.—The data presented in Table IV were compiled from several experiments in which blood lymphocytes were obtained from various combinations of isogenic strains of rats, placed into mixed cultures, and incubated over a 4–7 day period. These data are presented in a qualitative fashion and a positive (+) proliferative response with a particular combination of cells indicates that the peak reaction exceeded 300 cpm. In

| Group | Strain combination* | AgB genotype | Proliferative response |
|-------|---------------------|---------------|-----------------------|
| I     | BN + DA             | 3/3 + 4/4     | +                     |
|       | BN + L              | 3/3 + 1/1     | +                     |
|       | BN + F              | 3/3 + 1/1     | +                     |
|       | L + DA              | 1/1 + 4/4     | +                     |
|       | L + F               | 1/1 + 1/1     | −                     |
|       | BH + L              | ? + 1/1       | −                     |
|       | BH + BN             | ? + 3/3       | +                     |
|       | BH + DA             | ? + 4/4       | +                     |
|       | BH + F              | ? + 1/1       | −                     |
| II    | BN + BN/DA          | 3/3 + 3/4     | +                     |
|       | DA + BN/DA          | 4/4 + 3/4     | +                     |
|       | L + L/BN            | 1/1 + 1/3     | +                     |
|       | BN + L/BN           | 3/3 + 1/3     | +                     |
|       | L + L/DA            | 1/1 + 1/4     | +                     |
|       | DA + L/DA           | 4/4 + 1/4     | +                     |
|       | F + BN/F            | 1/1 + 3/1     | +                     |
|       | BN + BN/F           | 3/3 + 3/1     | +                     |
|       | F + L/F             | 1/1 + 1/1     | −                     |
|       | L + L/F             | 1/1 + 1/1     | −                     |
|       | BH + L/F            | ? + 1/1       | −                     |
|       | BH + BN/F           | ? + 3/3       | +                     |
| III   | L/DA + L/BN         | 1/4 + 1/3     | +                     |
|       | BN/F + L/BN         | 3/1 + 1/3     | −                     |
|       | BN/F + BN/DA        | 3/1 + 3/4     | +                     |
|       | L/BN + BN/DA        | 1/3 + 3/4     | +                     |

* BN, Brown Norway; L, Lewis; DA, DA; F, Fischer; BH, Black Hooded.
all cases, negative (−) responses were less than 50 cpm during this incubation period. For all these experiments, a 50:50 mixture containing a total of 2 million cells was employed in culture medium supplemented with 10% and sometimes 20% fresh BN rat serum.

Group I shows that positive proliferative responses were obtained whenever the donors were of different Ag-B genotypes. Combinations of cells from Lewis and Fischer animals—which are of the same Ag-B genotype—gave no evidence of proliferation. Also, combinations in which BH and Lewis or BH and Fischer were employed as donors did not undergo any response. This suggests that the Ag-B genotype of the BH rat, which has not as yet been determined by serologic means, is the same as L and F animals; namely, Ag-B1/1.

The nonreactivity of mixed cultures of lymphocytes from L and F, L and BH, and F and BH was somewhat surprising in view of the comparatively short survival times of skin homografts (8-10 days) exchanged between members of these strains. It seems, therefore, that under the culture conditions employed in these experiments, a proliferative response in mixed cultures of homologous rat lymphocytes occurs when the donors differ by one of the alleles at the AgB locus.

7. Unidirectional Culture Responses.—Activity observed in mixed cultures of cells derived from two different strains are presumably the outcome of two responses following mutual stimulation, strain A versus B and vice versa. It is therefore pertinent to investigate the extent of the response of mixed cultures of cells from parental and F1 hybrid animals. The data presented in Group II of Table IV show that proliferative responses do occur in cultures of parental and F1 hybrid lymphocytes, but again, only when there are isoantigenic incompatibilities ascribable to allelic differences at the Ag-B locus. The results of a typical experiment with hybrid and parental cells with various strain combinations is presented quantitatively in Fig. 8. It was expected (see reference 20) that the total uptake of tritiated thymidine, in terms of counts per minute per culture, of the two parental–F1 mixtures (p1 + F1 and p2 + F1) might be approximately equal to the activity of the parent-parent (p1 + p2) response. However, in no instance was this the case. In fact, when cells from DA and Lewis strains and their F1 hybrid were used, the maximal response in DA + L cultures was consistently lower than in cultures with DA + L/DA cells. With this particular strain combination, the reactivity of the DA + L/DA cultures was remarkably similar to that of the DA + L cultures for the first 5 days, but then DNA synthesis slowed down and ceased in the DA + L cultures while it continued in the cultures of DA + L/DA cells. The appearance of reactivity in parallel cultures of L + LDA cells consistently lagged behind that in the other cultures.

When L, BN, and L/BN hybrid animals were employed as donors, the pat-

15 Silvers, W. K. Personal communication.
tern of response was also consistent, but differed from that obtained with the other combinations. In this instance, reactivity of parent-parent cell cultures was similar to that of L + L/BN and significantly greater than that occurring in BN + L/BN cultures. Patterns of response when using DA, BN, and DA/BN F1 hybrid cells were again consistent, and differed from the others. With this combination, culture responses were particularly vigorous with all combinations of parental and F1 cells. Generally, however, the reactivity of BN + DA/BN cells was somewhat weaker.

It is interesting to note that the reactivity ostensibly of parental cells, was minimal in combinations with some hybrid cells (e.g., L + L/DA and BN + L/BN) and showed vigorous responses in other combinations (e.g., L + L/BN and BN + DA/BN).

Although it seems reasonable to assume that the stimulation of DNA synthesis which occurs in a parent-F1 mixture of cells is unidirectional, the evidence presented above certainly does not prove that this is the case. A more
direct means of examining this issue was to culture parent and F1 cells from donors of different sexes and subsequently to examine the sex chromosomes in mitotic figures found in the proliferating cultures. For a preliminary experiment, cells were obtained from DA males and DA/BN females and committed to culture under standard conditions. At various times, from the 5th to the 8th day of incubation, cultures were exposed to colchicine (5 \times 10^{-7} \text{M}) for a period of 4 hr prior to harvesting. The cells were fixed and chromosome preparations made according to methods described by Hungerford and Nowell (13). The ratio of mitotic figures per 1000 nucleated cells was determined microscopically by Dr. P. C. Nowell and the sex of the donor of the dividing cell ascertained from morphological differences in the X and Y chromosomes (14). The results from this single experiment (Table V) were clear-cut. Over a 5–8 day period, the mitotic index of proliferation in the mixed cultures of DA male and DA/BN female lymphocytes was 1.5–7.3%. Twenty-five random mitotic figures were examined from each of duplicate cultures harvested at daily intervals; all proved to be male, and therefore were derived solely from the DA parent. This finding constitutes strong direct evidence that with the strain combination employed (a) proliferation of mixed cultures of parent and hybrid cells is unidirectional at least for the first 8 days of incubation; (b) only the parental lymphocytes in the mixture are synthesizing DNA; and (c) even in an environment conducive to cell proliferation, cells from a hybrid donor do not respond to parental strain cells. Further studies are being conducted with other parental–F1 donor sex combinations to extend these observations to other strains, and to determine if, at any time after the first week of culture, hybrid cells may be responsive (see following paper, reference 29).

That cells from F1 donors are not innately unresponsive in culture is shown
by the data in Group III of Table IV. This summarizes the results of a group of experiments in which cells from unrelated F1 donors were committed to culture (e.g., P1/P2 + P1/P2).

From an examination of the results of culture mixes such as those presented in Fig. 8, it is possible to establish a rough order of reactivity of mixed cultures of cells from various strain combinations. Such a spectrum is easier to determine with mixed cultures of parental-hybrid cells, than with parental-parental cells, because the latter involve two-way reactions. However, for the sake of completeness, the parent-parent reactions are also ranked (see Table VI). Thus, cultures of DA + DA/BN or DA + BN cells are the most reactive and those of BN + L/BN are the least. Generally, this ranking is quite consistent. However, not infrequently, there may be little to distinguish between the reactivity of two adjacent combinations.

In considering the magnitude of the proliferative responses which result when various Ag-B combinations are tested, it can be seen that DA animals (Ag-B4/4) respond more strongly to Ag-B3 antigen than to Ag-B1; BN (Ag-B5/3) is more reactive to Ag-B4 than to Ag-B1; and Lewis (Ag-B1/1) is more reactive to Ag-B3 than to Ag-B4.

8. Attempts to Procure Unidirectional Reactivity with Mixed Cultures of Parental Cells.—Recent studies of Bach and Voynow (15) indicate that unidirectional or one-way responses are obtainable in mixed cultures of human lymphocytes by prior treatment of one of the donors' cells with mitomycin C. It was decided to investigate this phenomenon further with cells from parental strain and F1

| Combination                  | AgB alleles involved |
|------------------------------|----------------------|
| Most reactive: DA + DA/BN    | 3                    |
| (DA + BN)                    |                      |
| BN + DA/BN                   | 4                    |
| DA + L/DA                    | 1                    |
| L + L/BN                     | 3                    |
| (L + BN)                     |                      |
| (DA + L)                     |                      |
| L + L/DA                     | 4                    |
| Least reactive: BN + L/BN    | 1                    |

TABLE VI
Rank of Reactivity of Cultures of Mixed Lymphocytes from Various Rat Strain Combinations and their F1 Hybrids
rats. The fact that hybrid cells are "naturally" unreactive to parental strain cells allows one to determine whether or not a specific regimen of treatment designed to inhibit or abrogate proliferation in the "stimulator" population has any effect on their capacity to act as a mitogen to the responding population of cells.

![Graph showing the influence of mitomycin C on proliferative and stimulatory activities of parental and F1 cells in the mixed lymphocyte interaction.](image)

**Fig. 9.** Influence of mitomycin C on the proliferative and stimulatory activities of parental and F1 cells in the mixed lymphocyte interaction. Parental cells pretreated with mitomycin C (-- --), F1 cells pretreated with the drug (-----), no pretreatment (---).

For these experiments, peripheral blood lymphocytes were obtained from parental and F1 hybrid animals as above and adjusted to concentration of 10 million/ml. 5 ml aliquots were incubated for 20 min at 37°C with or without mitomycin C (final concentration, 25 μg/ml) in medium containing 10% fresh BN serum. Following this incubation period, the cells were washed twice in medium (without serum), adjusted to an appropriate concentration after counting, and then committed to culture under the standard conditions. The results of one experiment with L, BN, and L/BN cells are fully typical of other experiments conducted with other strain combinations (see Fig. 9). It is clear that
pretreatment of both parental cells with mitomycin C completely obliterates reactivity in parental–parental cultures, and markedly diminishes the response in parental–F₁ cultures. An unexpected finding, however, was that pretreat-

**TABLE VII**

*Summary of Experiments with Mixed Cultures of Rat Lymphocytes in Medium Supplemented with Serum (10–20%) Prepared from Rats of Different Strains*

| Serum     | Cell mixtures tested                                      | Proliferative response |
|-----------|----------------------------------------------------------|------------------------|
| BN        | All combinations listed in Table IV                     | +*                     |
| Lewis     | BN + L                                                   | –                      |
|           | BN + DA                                                  | –                      |
|           | BN + F                                                   | –                      |
|           | L + L/DA                                                 | –                      |
| Fisher    | BN + F                                                   | –                      |
|           | BN + L                                                   | –                      |
| DA        | BN + L                                                   | +                      |
|           | BN + DA                                                  | +                      |
|           | L + DA                                                   | +                      |
|           | F + L                                                    | –                      |
| L/BN      | L + BN                                                   | +                      |
|           | L + L/BN                                                 | +                      |
|           | BN + L/BN                                                | +                      |
|           | DA + DA/BN                                               | +                      |
| L/DA      | DA + BN                                                  | +                      |
|           | L + L/DA                                                 | +                      |
| BN + Lewis (50:50) | DA + BN                                           | +                      |
|           | BN + L                                                   | +                      |
|           | L + L/BN                                                 | +                      |
|           | BN + L/BN                                                | +                      |

* All combinations that differ by an allele at the AgB locus.

ment of F₁ hybrid cells with the drug drastically reduced subsequent reactivity of untreated parental cells in mixed parental–hybrid cell cultures. It must be assumed, therefore, that prior treatment of parental cells from one of the donors of mixed parental–parental cell cultures with mitomycin C has two effects: (a) direct inhibition of DNA synthesis by the treated parental cell population, and (b) an indirect inhibition of the response of the untreated parental cell population—perhaps due to the failure to remove all the mitomycin C prior to culturing.
For this reason, it would be risky to draw any conclusions based on quantitative differences of response when the culture material includes cells that have been pretreated with this drug. On the other hand, interpretations based simply upon the presence or absence of a proliferative response would not be invalidated by this "side-effect" of mitomycin C treated cells.

Attempts to circumvent this difficulty by the use of lower concentrations of the drug and shorter periods of incubation pretreatment met with no success. While the responding population was not as severely affected by such pretreatment, DNA synthesis by the treated cells was also not completely abolished.

9. Studies on the Capacity of Rat Serum to Support the Proliferative Response.—During the course of this study, it became obvious that sera harvested from rat donors of various strains differed markedly in their capacity to support the proliferative response in a mixed lymphocyte culture. When sera from BN, DA, L/DA, or L/BN animals were used, mixed cultures of cells from donors of different Ag-B genotypes gave consistently positive responses (see Table VII). Responses with Lewis or Fischer serum were either poor or absent. It was clear that the nonreactivity of cultures supplemented with Lewis or Fischer serum was not due to an immunologic incompatibility between the cells and the sera, since mixed cell cultures of L + L/BN or BN + L/BN cells were consistently negative in Lewis serum, but positive in BN serum.

It was also noticed that the capacity of sera from different strains of rats to support the proliferative response was generally all or none. Fig. 10 shows the curve of response of mixed cultures of L + BN cells supplemented at a concentration of 20% with serum from various rat strains. There was little quantitative difference in the supportive capacity of serum from BN, L/BN, or DA donors. Furthermore, that serum from L/BN donors, as well as 50:50 mixtures of sera from L and BN donors, is quantitatively as effective in supporting the synthesis of DNA in mixed cultures suggests that the inability of Lewis or Fischer serum to do so is probably not due to the presence of an inhibiting or toxic substance, but more likely to the absence of some essential component.

It was possible that the inability of Lewis or Fischer serum to support DNA synthesis was related to their AgB1/1 genotype. This possibility was tested by the use of a backcross population of animals derived from L/DA × L matings. Parallel cultures of lymphocytes from each of these animals were mixed in culture with parental Lewis cells, and serum from each of these backcross donors was used to supplement cultures of L + L/DA cells. Since these backcross donors have a complete set of Lewis isoantigens and a variable number of DA factors, some of them can be expected to be Ag-B1/1 and others Ag-B1/4.

The Ag-B genotype of these backcross animals can be determined by serologic means, and Silvers, Wilson, and Palm (16) have recently shown that Lewis cells (1/1) selectively respond to the incompatible 1/4 backcross donor cells in mixed cultures. If the capacity to support DNA synthesis in mixed lymphocyte cul-
tures is associated with the AgB-4 genotype, then serum, obtained from those backcross donors whose cells elicit a positive proliferative response in cultures with Lewis cells, should support proliferation in mixed cell cultures.

The results of this experiment are presented in Fig. 11, and show that a panel of 10 backcross animals, lymphocytes from 5 of them (♀ 1, 3; ♂ 1, 2, 3), mixed individually in cultures with Lewis cells elicited positive responses on the 6th day of incubation and therefore were presumably of genotype 1/4. Nevertheless serum from some of these 1/4 animals were not able to support DNA synthesis in mixed cultures of L + L/DA cells. This suggests that the ability of sera to support in vitro reactions is not related to the Ag-B locus and is probably inherited independently.

Further studies were conducted to determine some of the conditions under which serum from BN animals would support mixed lymphocyte proliferative responses. Fig. 12 shows the results of experiments in which cells from DA and DA/BN animals were cultured for various times in media containing fresh
Fig. 11. Comparison of the capacity of blood lymphocytes from various L/DA x L backcross donors to stimulate a proliferative response of Lewis cells (in culture with BN serum) with the capacity of sera from these backcross donors to support proliferation in L + L/DA cultures. Cultures harvested on day 6.
BN serum at various concentrations. As can be seen, there was little difference in the effect of the various concentrations over the range 5-20%. However, at 40% the proliferative response of the cells was somewhat decreased.

![Graph showing the influence of various concentrations of BN serum on response of mixed DA + DA/BN cultures.](image)

**Fig. 12.** Influence of various concentrations of BN serum on response of mixed DA + DA/BN cultures.

Fig. 13 shows the results of pretreating BN serum by various means on the response of DA + DA/BN cultures. A pool of serum from BN animals was prepared and this was subdivided into aliquots which were preincubated at 37°C, stored at 4°C or −20°C for 6 days, or lyophilized. These various samples were then thawed or reconstituted with an appropriate volume of sterile distilled...
water, and their effectiveness in supporting the proliferative response of DA + DA/BN cell cultures (at a concentration of 10%) was compared to aliquots of fresh BN serum that had been heated to 56°C for 30 min or left untreated. The results indicate that there was little to choose between the various treatments of sera, except that samples previously incubated at 37°C for 6 days were clearly less effective.

![Graph showing the effect of various pretreatments on the capacity of BN serum to support proliferative activity of DA + DA/BN cultures.](image)

**DISCUSSION**

Numerous substances—having little else in common—are known to stimulate a blastogenic response and mitotic activity on the part of peripheral blood leukocytes in culture. Most of the evidence, although circumstantial, suggests that it is the small lymphocyte which reacts to these agents, i.e., positive responses occur with culture inocula consisting almost entirely of small lymphocytes (17–19) (for reviews, see references 20, 21, 22).

These mitogenic substances can be classified into one of three groups according to certain parameters of the proliferative responses they provoke. **First**, nonspecific
agents such as phytohemagglutinin (PHA) (17-19), Pokeweed mitogen (PWM) (23), anti-leukocyte (24), and anti-allotype (25) sera induce a response involving the vast majority of the cell population committed to culture, and do so in a relatively short period of time (3–4 days).

Second, mitogens such as the purified protein derivative of tuberculin (2, 3), induce a blastogenic response of smaller magnitude (10–30% blast cells) and one which is somewhat more delayed in onset (4–5 days). These substances have been termed “specific mitogens” since only lymphocytes from previously sensitized donors will respond to the substances in culture. Furthermore, because these agents were all good antigens, it was believed by many that the proliferative responses they induced might have an immunologic basis—and represent a recognition reaction in vitro of antigens previously encountered in vivo. Indeed Mills (26) has shown that the response of guinea pig lymphocytes to tuberculin occurs only with donors in which a state of delayed hypersensitivity had been induced. Cells from animals with immediate hypersensitivities having high titers of circulating antibody were not reactive in culture. Some workers have considered that the “nonspecific mitogens,” PHA, etc., might also be antigens, and that the cellular response to these agents in culture is an immunologically specific one. This view has not received wide acceptance primarily because cells from most species are known to respond to PHA, and it is unlikely that all these cell donors were sensitive to this agent as an ubiquitous antigen. Also, it is difficult to visualize how the response to PHA could involve the vast majority of cells that it does if it has an immunologic basis.

Finally, the proliferative, blastogenic response of human lymphocytes to cells and cell products of unrelated donors was first described by Bain and her colleagues (6). The responses in these “mixed lymphocyte interactions” are more delayed in onset (5–8 days) than in the other two groups, of smaller magnitude (5–10% blast cells), and are further distinguished from the “specific responses” in that they do not require the prior sensitization of the cell donors. Further work by Bain and her group (27) and by others (7) has shown that the degree of response is directly related to the immunogenetic disparity of the co-donors. Cultures from monozygotic twins do not undergo proliferation; only slight responses occur with cells from closely related donors; and maximal responses result when unrelated donors are employed. It is on this basis that many contend that mixed lymphocyte cultures may provide a useful typing procedure for the selection of histocompatible donor/recipient combinations in surgical programs of tissue and organ transplantation.

Dutton (see review, reference 20) and his co-workers have extended these studies on the mixed lymphocyte interaction to culture material consisting of splenic or lymph node suspensions from outbred rabbits and from different isogenic strains of rats or mice. In an effort to define more precisely the role of genetically determined factors, he showed that mixtures of splenic cells from mice of the same strain did not respond in culture, whereas positive responses were obtained with most donor combinations involving different inbred strains. Dutton suggested, with due caution, that the proliferative response of mixed cultures of antigenically disparate lymphocytes might have an immunologic basis and be directed against histocompatibility antigens.
The results of experiments presented in this communication provide strong support for this contention. **First**, proliferative responses occur in mixed cultures of peripheral blood leukocytes obtained from rats of disparate isogenic strains which differ with respect to important histocompatibility antigens determined by alleles at the Ag-B locus. Mixed cells from donors of inbred strains which possess common antigens determined by this locus show no response in culture. Bach and Amos (28) have recently reported on the results of studies which show that the proliferative response of human lymphocytes in the mixed lymphocyte interaction depends on the presence or absence of antigens determined also by a single locus.

**Second**, within isogenic stocks of animals, the immunologic nonreactivity of F1 hosts challenged with tissues or cells from parental strain donors is well known. This follows from the fact that histocompatibility antigens are determined by co-dominant genes and therefore the complement of histocompatibility factors of both parents is present in F1 individuals. If the mixed lymphocyte interaction represents an immunologic response at the cellular level, then F1 cells should not respond to cells of parental origin in culture. Examination of mitotic figures in mixed cultures of cells from parental and F1 donors of different sexes, using the X and Y chromosomes as morphological markers, shows that the proliferative responses in parental–F1 cell cultures is indeed unidirectional. All of the mitotic figures were determined to be of parental origin. Therefore, at least on the basis of this preliminary experiment, even in an environment conducive to blastogenic transformation and cell proliferation, cells from a hybrid donor do not respond to antigens of parental cells. (These studies have been extended to different parental–F1 combinations, and their results are presented in the following paper (29).

**Third**, the recent studies of Silvers, Wilson, and Palm (16) provide strong evidence that the factor responsible for stimulating cells in culture are transplantation antigens determined by the Ag-B locus or, by a closely linked genetically determined factor. This follows from their findings that parental Lewis lymphocytes reacted in culture against cells from backcross donors (Lewis × Lewis/DA) that were determined serologically to possess the incompatible allele at the Ag-B locus.

**Fourth**, the fact that responsive cells in a mixed culture of leukocytes, lymph node, or splenic cells are apparently lymphocytes further supports the premise that the mitogenic response of a mixed lymphocyte interaction has an immunologic basis since it is these cells that are directly involved in immune mechanisms in vivo.

It should be pointed out that, although it is strong, the evidence that lymphocytes are the responsive cells in in vitro culture systems is not conclusive. This has been recently stressed by Dutton (20). The experiments presented here
show that cells obtainable from the circulating lymph (thoracic duct) are capable of response to homologous cells, and this implicates the small lymphocytes primarily, but does not prove the point. Furthermore, it has yet to be established whether the cells arise by a morphological transformation of a significant portion of the initial population or by a rapid and continuous proliferation of a very small fraction of the cultured population.

Whether or not small lymphocytes are the responsive cells has important implications. For example, this would suggest that some of the initial stages in the development of immune responses do not require the participation of more than one cell type. It must be borne in mind, however, that during the 3 or 4 days' incubation period before the mitogenic response, other cell types filling participatory roles may arise from the initial culture inocula. Therefore, it is pertinent that the addition of peritoneal exudate cells, and presumably some macrophages, does not alter the magnitude or kinetics of the mixed lymphocyte interaction.

The kinetics of the mixed lymphocyte interaction as reported by Dutton (30) for cultures of splenic and lymph node lymphocytes are fundamentally different from those presented here. Thus, Dutton measures the proliferative responses in mixed cultures of homologous rat splenic cells in cultures terminated during the second day of incubation, whereas with the present studies, significant levels of incorporation of radioactive thymidine did not occur until the 4th day or later. These kinetics moreover, are similar to those repeatedly shown for the response of mixed cultures of human leukocytes (9, 15). The disparity between these and Dutton's results might be explained by the difference in origin of the cultured lymphocytes—one being the peripheral blood and the other the spleen. The behavior of these two populations can be considered to be quite different—especially in view of the heterogeneity of cell types as well as a physiological heterogeneity among lymphoid cells present in the spleen.

If the mixed lymphocyte interaction is interpretable as a preliminary phase of a primary immunologic response at the cellular level, it is initiated by cells outside the architectural framework of the regional lymph node. A natural extension of this argument from this tissue culture system is that, when confronted with a transplantation antigen in vivo, cells of the lymphoid series are capable of initiating immune responses and executing them in situ. This implies that competent lymphoid cells can be committed to the status of immunologic effector cells without undergoing a period of residence within a draining lymph node for the purpose of proliferation or maturation. The validity of this premise has already been established by the demonstrations that immunologically competent homologous lymphocytes from normal nonimmune donors seeded onto the chorioallantoic membranes of chick eggs (31) or inoculated beneath the renal capsules of rats can produce lesions as evidence of their im-
munologic capabilities (32). For the development of a systemic immunity (demonstrable upon subsequent challenge with a specific antigen) however, involvement of lymphatic tissue may be obligatory.

SUMMARY

Some of the conditions and parameters of the proliferative response in the mixed lymphocyte interaction have been studied with the use of culture inocula consisting of lymphoid cells from various immunogenetically diverse, isogenic strains of rats. Procedures are described by which consistent culture responses can be obtained which are measurable in terms of incorporation of radioactive thymidine into a trichloroacetic acid precipitable cell fraction. Results of experiments with this "mixed lymphocyte interaction" show that

(a) Incorporation of thymidine into the mixed cultures is detectable only during and after the 4th day of incubation. Use of culture inocula derived from the thoracic duct or partially purified suspensions of lymphocytes from the peripheral blood provides further evidence that it is the small lymphocyte which synthesizes DNA in these cultures. The addition of peritoneal exudate cells, presumably containing some macrophages, to the mixed lymphocyte cultures does not alter the kinetics of the response.

(b) Proliferative responses occur in mixed cultures of cells only when the donors differ by alleles at the important Ag-B histocompatibility locus.

(c) Proliferative reactivity in mixed cultures of lymphocytes from parental and hybrid donors is unidirectional. With the use of chromosomally marked cells from donors of different sexes, it was established that only parental lymphocytes are dividing in mixed cultures of parental and hybrid cells.

The results of these experiments strongly support the premise that the proliferative response in mixed cultures of lymphocytes represents a de novo immunologic reaction on the cellular level against histocompatibility antigens.

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