Mixed Leukocyte Stimulation of Normal Peripheral Leukocytes by Autologous Lymphoblastoid Cells

Abstract. *Lymphocyte culture lines can be established by using buffy coat cells from normal or diseased individuals. Established lymphocytoblasts from a normal donor were used in the one-way mixed leukocyte culture test and were found to stimulate the peripheral lymphocytes of that donor. These lymphocytoblasts were also capable of stimulating allogeneic lymphocyte populations.*

Investigations have shown that lymphocytes from normal human donors can be stimulated by a number of varied agents, including phytohemagglutinin, tuberculin, pokeweed mitogen, $HgCl_2$, staphylococcal filtrate, and homologous lymphocytes (*1*). Once stimulated, significant numbers of cells transform within a week to pyroninophilic blast cells that synthesize nucleic acids and undergo cell division. In certain cases, however, lymphocytes maintained in culture without the aid of an added mitogenic agent may divide and from these cells lymphoblastoid cell lines have been established (*2*).

Fridman and Kourilsky (*3*), utilizing the one-way mixed leukocyte culture test (MLC), demonstrated that peripheral lymphocytes can be stimulated in culture by autologous leukemic lymphoblasts. We report here that normal peripheral leukocytes can be stimulated by the established autologous lymphoblast cell culture.

The following procedures were used for the establishment of the lymphoblast cultures. One hundred milliliters of freshly drawn, heparinized blood (10 units of heparin per milliliter) was centrifuged at room temperature for 10 minutes at 60*g*. The leukocyte-rich plasma was removed. Upon additional settling, another 5 ml of leukocyte-rich plasma was obtained. Both samples were pooled and centrifuged at 650*g*, the plasma was removed, and the cell pellet was resuspended in sterile Hanks balanced salt solution (Grand Island Biological). Cell counts were performed and cultures were initiated with a cell concentration of 3.5×10^6 leukocytes per milliliter. The medium employed was RPMI 1640 (Grand Island Biological; RPMI, Roswell Park Memorial Institute) supplemented with fetal calf serum, 16.6 percent; glutamine, 2 mmole/liter; and neomycin, 80 μg/ml. All cultures were maintained statically at 37°C. Every 48 to 72 hours additional fresh medium was added (10 percent by volume); at the time of each fifth addition to the medium (10 to 15 days) viable cell counts were made with the use of trypan blue dye exclusion, and 80 percent of the old medium was replaced with fresh medium. This procedure essentially followed that of Moore *et al.* (*4*); it was continued until the culture became established, at which time the designation was NMRI 2666. A second culture derived from 2666 was adapted to pooled human serum (blood group A, Rh positive), 16.6 percent concentration in the above medium, and designated NMRI 2748.

In the mixed leukocyte test as performed in this laboratory, peripheral blood leukocytes from a given donor (A) are mixed with stimulating cells that have been mitotically inhibited by pretreatment with mitomycin C (*5*). These cells are either autologous lymphoblasts (B) or lymphocytes from an unrelated individual Xm (m denotes mitomycin C treatment). The untreated leukocytes are assessed to determine whether they have been stimulated by the presence of cells treated with mitomycin C by using the uptake of [^{3}H]thymidine into the trichloroacetic acid (TCA) precipitable material. An increased incorporation of [^{3}H]thymidine shows stimulation of the A cells by the mitomycin C–treated cells that are present. If the cells used in the reaction are identical, the cell mixture ABm will not incorporate [^{3}H]-thymidine at a rate different from their respective controls (AAm, Bm).

In each of the nine reactions employing donor cells (A) and the autologous cultured cell line (B) there was stimulation as determined by the [^{3}H]-thymidine found in the TCA precipitable material. Stimulation was also apparent when allogeneic cells were used with the lymphoblast cell line (Table 1).

In experiment 1 (Table 1) control cultures of cells from individual A incubated with Am (treated with mitomycin C) incorporated 750 count/min. When mixed with autologous lymphocytoblasts (Bm; treated with mitomycin C) there was a clearly increased uptake of [^{3}H]thymidine at the cell concentrations employed. Mitomycin C–treated lymphoblasts incorporated significantly less or no [^{3}H]thymidine when compared to lymphoblasts B that were

Table 1. Experiment 1 is a study of peripheral leukocytes of donor (A) and the lymphocytoblast culture B (2666 treated with mitomycin C). A second unrelated individual (X) is also utilized in the procedure as an additional control. Responding cell concentrations are 0.5×10^6 mononuclear cells per milliliter for both individuals A and X. Stimulating cell concentrations are total lymphoblasts per milliliter or total lymphocytes per milliliter. Mixed cultures are performed as described (*5*). Cultures were incubated for 3 days at 37°C in a humid atmosphere, and, on day 3, 2 μc of [^{3}H]-thymidine was added to each tube for 18 hours. Counts per minute refers to the average for washed acid precipitates (*8*) of triplicate cultures.

Cell mixture	Concentration of stimulating cells (No. $\times 10^6$/ml)	Activity (count/min)
AAm		750
ABm	0.5	14,611
ABm	1.0	10,285
ABm	1.5	3,412
B		132,176
Bm	0.5	90
Bm	1.0	118
Bm	1.5	47
AXm	0.5	4,201
AXm	1.0	6,471
AXm	1.5	7,496
XXm		938
XAm	0.5	5,258
XAm	1.0	4,412
XAm	1.5	5,290
XBm	0.5	10,363
XBm	1.0	15,750
XBm	1.5	12,039

Table 2. Experiment 2 is the study of lymphoblasts cultured in 16.6 percent fetal calf serum and of these same cells adapted to grow in medium containing 16.6 percent type A, Rh positive human serum. Peripheral lymphocytes of donor A were tested with stimulatory cells B (fetal calf serum culture) and C (pooled type A, Rh positive human serum culture). Both lymphoblast lines were treated with mitomycin C. Cell concentrations and culture conditions as in Table 1.

Cell mixture	Concentration of stimulating cells (No. $\times 10^6$/ml)	Activity (count/min)
AAm		603
ABm	0.5	29,172
ABm	1.0	10,394
ABm	1.5	2,908
B		156,406
Bm		388
ACm	0.5	24,274
ACm	1.0	19,387
ACm	1.5	14,190
C		283,125
Cm		992

not treated with mitomycin C (132,176 count/min as compared to the control of 90 count/min). The allogeneic cells (X) used as stimulating cells verified the ability of A to respond, and the reciprocal test was also shown to be stimulatory (XAm). The allogeneic lymphocytes in the reaction XBm also incorporated [^{3}H]thymidine when incubated with the lymphoblast (Bm).

One possible explanation of the stimulation caused by autologous lymphocytoblasts is that fetal calf antigens derived from the culture medium had become associated with the cultured cells. To test this possibility, lymphoblast culture 2666 was washed twice with medium containing human type A, Rh positive serum and then was cultured in medium in which human serum (same ABO and Rh type as original donor) had been substituted for the fetal calf serum. After 30 days, the lymphoblasts grown in medium containing human serum were assayed for stimulatory activity in the mixed leukocyte culture test (Table 2). These lymphoblasts also stimulated the autologous peripheral lymphocytes (24,214 count/min compared to controls of 603 and 992 count/min), which suggests that antigens derived from fetal calf serum had played no role in the capacity of these cells to stimulate. Furthermore, we were unable to detect bovine antigens on these cells in cytotoxicity tests employing antiserum to fetal calf serum and antiserum to Forssman antigen.

Another possible explanation of the stimulation caused by autologous lymphoblast cells is that the cells elaborate a soluble factor that causes stimulation of the peripheral leukocytes. An attempt was made to determine if the cell-free culture filtrate was stimulatory. No stimulation was found when three dilutions of the culture filtrate were used (2.0, 1.0, and 5.0 ml of filtrate compared to fresh medium; all volumes were adjusted to 2.5 ml with the medium employed in the MLC). This result suggests that a soluble blastogenic factor is not present, but of course such experiments do not rule it out entirely.

The autologous stimulation described here is comparable to the stimulation observed in mixed cultures of lymphocytes from allogeneic individuals possessing different alleles at the HL-A locus. Using methods routinely employed in this laboratory (*6*), we established that there is no difference between the HL-A type of the donor and the HL-A type of his autologous lymphoblast cultures. These observations are in agreement with those of Kourilsky *et al.* (*7*).

The remaining alternative interpretation of this phenomenon is that cells in culture synthesize an antigen not found on the peripheral lymphocyte. Bach *et al.* (*5*), using leukemic cells, suggested that the stimulation found in their laboratory may be due to a leukemic antigen, minor non-HL-A antigens, or a blast cell antigen. It appears from our data that the reaction may be caused by a blast-associated antigen that is not found on normal lymphocytes. Further investigations will be necessary to elucidate the mechanism of this in vitro reaction of autologous cells. Such investigations may be expected to facilitate understanding of the MLC test and its relationships to immunological events.

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10. Since the submission of this report, the work of C. M. Steel and D. A. Hardy [*Lancet* **1970-I**, 1322 (1970)] has come to our attention. They present similar findings using cells cultured from patients with infectious mononucleosis. Our observations first reported here concern the reactions of a cell line derived from a clinically healthy normal donor.

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Starvation in Human Pregnancy: Hypoglycemia, Hypoinsulinemia, and Hyperketonemia

Abstract. *In women fasted during the second trimester of pregnancy, concentrations of glucose and insulin in the plasma fell to a greater extent and ketone acid concentrations in the blood rose more rapidly than in nonpregnant controls. Nitrogen excretion in the urine, particularly ammonia, was increased in the pregnant group. Continuous glucose utilization by the conceptus may exaggerate and accelerate the metabolic consequences of starvation.*

The influence of pregnancy on maternal metabolism has been extensively investigated in human subjects in the postabsorptive and fed states (*1*). However, the metabolic response to periods of fasting extending beyond 12 to 15 hours, although well characterized in nonpregnant humans (*2*) and intensively studied in pregnant rats (*3*), has not been examined in human pregnancy. In view of the obligate requirements of the fetus for glusose (*4*), the changing hormonal milieu characteristic of pregnancy (*5*), and species differences in maternal and fetal metabolism (*4*), observations on nonpregnant individuals or on pregnant laboratory animals may not be applicable to the human gravid state. Furthermore, the potential importance of maternal undernutrition in the genesis of alterations in fetal growth and development (*6*) underscores the need for characterizing the maternal response to caloric deprivation. In our study the metabolic response to an 84-hour fast has been studied in women during the second trimester of pregnancy and compared to that of a nonpregnant control group.

The pregnant subjects were 12