AUTOLOGOUS STIMULATION OF HUMAN LYMPHOCYTE SUBPOPULATIONS*

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One basic attribute of lymphocytes is their ability to recognize self from nonself. Lymphoblastoid transformation and the associated \(^{3}H\)thymidine incorporation in in vitro cultures of lymphocytes is generally recognized as a response to nonself antigens. We report here on the surprising finding that in vitro stimulation occurs among autologous lymphocytes in the mixed lymphocyte culture (MLC) test. The most likely explanation of our findings is that among lymphocytes obtained from one sample of peripheral blood, thymus-derived (T) lymphocytes respond against non-T cells in the autologous mixed lymphocyte culture (AMLC) reaction.

Materials and Methods

**Lymphocyte Fractionation.** Peripheral blood lymphocytes containing less than 5% granulocytes were isolated by Ficoll-Hypaque gradient centrifugation. The blood donors were healthy volunteers between 20 and 30 yr of age. Assessment of T cells and bone-marrow-derived (B) cells and fractionation of lymphocyte suspensions into T-rich and B-rich subpopulations by the sheep red blood cell (SRBC) rosetting technique and by goat-antihuman immunoglobulin column fractionation was done as described (1). Whereas unfractionated lymphocyte suspensions contained 70 ± 2% (mean ± SE) T cells and 19 ± 2% B cells, the respective percentages after fractionation by the SRBC rosetting technique in 11 experiments were 92 ± 1% and 6 ± 1% for the T-rich fractions and 29 ± 5% and 51 ± 4% for the B-rich fractions. Passage of lymphocytes through goat-antihuman immunoglobulin-coated columns did not increase the percentage of T cells equally well (80 ± 2%), but reduced the percentage of B cells to 4 ± 1%.

**Mixed Lymphocyte Culture.** MLC were done as described by micromethods using \(5 \times 10^4\) responding cells and \(5 \times 10^4\) irradiated stimulating cells in a total volume of 0.1 ml (2). All cultures were done in triplicate. Uptake of \(^{3}H\)thymidine was measured by liquid scintillation spectrophotometry, and counts per minute given are the mean cpm of the triplicate cultures ± SE. Stimulation ratios were calculated by dividing the mean counts per minute of a given experiment by the mean counts per minute of the responding cell's background control culture.

**Results**

The general outline of the experiments was to recombine the separated T-rich and B-rich fractions of lymphocytes in a checkerboard fashion. One representative experiment is given in Table I. In the first line, it can be noted that the unfractionated lymphocytes respond strongly to autologous B-rich cells. The
Autologous Mixed Lymphocyte Culture

Responder | T-cells/ B-cells | Stimulator (irradiated) | Allogeneic control (unfractionated) |
|-----------|----------------|-------------------------|------------------------------------|
|           | %              | Unfractionated | B-rich | T-rich | cpm ± SE ratio | cpm ± SE ratio |
| Unfractionated | 79/20         | 222 ± 27 | 3,401 ± 108 | 323 ± 108 | 6,718 ± 363 |
| B-rich     | 17/49          | 1,649 ± 207 | 1,536 ± 216 | 668 ± 183 | 4,833 ± 591 |
| T-rich     | 93/6           | 745 ± 120 | 2,833 ± 383 | 98 ± 4 | 6,089 ± 327 |
| Allogeneic control | 72/21    | 5,010 ± 429 | 8,368 ± 373 | 1,884 ± 339 | 230 ± 3 |

Unfractionated, B-rich and T-rich autologous lymphocytes were reacted against each other, as well as against allogeneic control lymphocytes, in checkerboard fashion. B-rich cells can be seen to significantly stimulate autologous unfractionated and T-rich cells. In contrast, T-rich responders are also stimulated by unfractionated cells, however, to a lesser degree. [3H]thymidine incorporation was the measure of stimulation. cpm ± SE and stimulation ratios are given.

The magnitude of this response (stimulation ratio of 15.35 and 3,401 cpm) is about half of the response to allogeneic cells (ratio of 30.31 and 6,718 cpm). The "background" of B-rich cells is high (1,536 cpm), whereas that of T-rich cells is extremely low (98 cpm). The T-rich fraction can be noted to be stimulated very well by the B-rich fraction (ratio 29.06) and not as well by autologous unfractionated cells (ratio 7.64). As stimulators of allogeneic unfractionated lymphocytes, B-rich cells are again more effective, whereas T-rich cells are poor stimulators.

Similar findings were seen in the 11 separate experiments as summarized in Fig. 1 and 2. The background of the unfractionated lymphocytes gave a mean cpm of 763 ± 125, and this was decreased in the T-rich fractions to 155 ± 33 and increased in the B-rich fractions to 3,470 ± 599. Invariably, T cells reacted against autologous B-rich stimulators with a stronger response (mean ratio 23 ± 5) than against unfractionated autologous cells (mean ratio 9 ± 4) (Fig. 1). The response of T-rich cells against allogeneic unfractionated lymphocytes gave a mean ratio of 69 ± 19. When irradiated T-rich cells were added to autologous B-rich responders, a significant reduction of the high background [3H]thymidine incorporation of B-rich cells was consistently observed (Fig. 2). The mean ratio in the 11 experiments was 0.39 ± 0.01.

The capacity to stimulate allogeneic responding lymphocytes was strikingly different for the various fractions. Whereas unfractionated lymphocytes produced a response with a mean ratio of 14 ± 2, B-rich cells stimulated significantly stronger (mean ratio 25 ± 4). T-rich cells were poor stimulators (mean ratio 8 ± 3), thus confirming previous observations by others and by us (1, 3).

In an additional series of eight experiments the T-rich cells obtained by goat-antihuman immunoglobulin column fractionation were tested in a similar manner. The background cpm of the T-rich fractions was 123 ± 23 and that of the unfractionated cells 1,158 ± 187. Autologous stimulation of T-rich responders by unfractionated stimulators was observed in each instance with a mean stimulation ratio of 12 ± 5. The results of AMLC with cells fractionated by both methods are thus comparable. Because the counts per minute obtained with T-rich
responders increased consistently in the following order: T-rich < unfractionated < B-rich stimulators, it appears plausible that B lymphocytes are the stimulators in the AMLC reaction. However, since the B-rich cell fractions also contained cells without surface markers, B cells were not positively identified as the stimulators.

We were careful to eliminate the possibility that inadequate inactivation of B-rich stimulators contributed falsely to the autologous stimulation of T-rich cells. Irradiated B-rich cells consistently gave an insignificant background of <100 cpm and could not be stimulated by allogeneic inactivated lymphocytes. Treatment of stimulating cells with irradiation or with mitomycin C was equally effective. B-rich cells disrupted by treatment with ultrasound were unable to stimulate autologous or allogeneic lymphocytes. Furthermore, no significant stimulation of freshly prepared T-rich cells was seen when supernates of autologous B-rich cells obtained after 5 days incubation at 37°C were added. These experiments suggest that membrane antigens rather than soluble factors are responsible for stimulation in the AMLC reaction. SRBC were unable to elicit a response when they were added to responding lymphocytes. Goat-antihuman
FIG. 2. B-rich lymphocytes give a strikingly higher "background" [\(^{3}H\)thymidine uptake (B-rich vs. B-rich) compared to unfractionated cells (unfractionated vs. unfractionated). Whereas addition of unfractionated stimulators to B-rich responders in most instances produced little or no change in [\(^{3}H\)thymidine uptake compared to background of B-rich cells, there was a significant decrease with T-rich stimulators. Lines connect test results obtained with cells of one donor.

immunoglobulin eluted from fractionation columns cannot account for autologous stimulation because the stimulator cells were not passed through columns, and the similar results obtained by both fractionation methods strengthen the validity of our study.

Discussion

The commonly observed background [\(^{3}H\)thymidine uptake in MLC has been shown to be drastically alterable by changing the proportion of T and B cells in the autologous lymphocyte preparation. Reduction of B cells results in lowering of the background and increase of B cells in heightening of the background. Although the background has been attributed in the past to stimulation by various ingredients of the culture media, it has been shown here that with a
constant media and variation of T- and B-cell proportions, the autostimulation is markedly influenced.

Despite the difficulties in obtaining absolutely pure T and B lymphocytes by the currently available techniques, even with T-rich and B-rich fractions, it is possible to infer that the T cells are the responding cells in the AMLC reaction. Removal of B cells lowers the stimulation and increasing the B cells increases stimulation, indicating that B cells are the stimulator cells (Fig. 1). The intermediate stimulation of T-rich responders by unFractionated stimulators is consistent with this assumption because the content of B lymphocytes in unFractionated cells is intermediate in comparison to T-rich and B-rich cells. The possibility that other cells such as null cells or monocytes could have contaminated the B-cell preparations cannot be excluded. The extremely high background count of B-rich cells in comparison to unfractionated cells (Fig. 2) is interpreted to be produced by the concentration of stimulator cells in this fraction.

Of great interest is whether autostimulation occurs in vivo. A certain level of spontaneous incorporation of [3H]thymidine can be found in freshly prepared lymphocytes. These activated cells were believed to be the result of immune reactions, such as rejection of kidney allografts (4), but were subsequently shown to fluctuate considerably, particularly after corticosteroid administration (5). The occurrence of autoantibodies against lymphocytes under a variety of conditions (6, 7) indicates that autostimulation in vivo is not impossible. Recently, in vitro autostimulation between peripheral blood lymphocytes and thymus cells in patients with myasthenia gravis was reported, and an abnormally high number of B cells was found in the thymus cell preparations of myasthenic patients (8). In healthy individuals the degree of in vivo autostimulation appears to be low. We found a significant increase in [3H]thymidine uptake between the 1st and the 5th day of the in vitro AMLC cultures. This suggests the existence of a regulatory mechanism in vivo. Serum factors similar to those suggested by Wekerle et al. (9) or active suppression of autostimulation by T cells are possibilities. The reduction in [3H]thymidine uptake when B-rich responders vs. B-rich stimulators as compared to B-rich responders vs. T-rich stimulators (Fig. 2) could be an indication of a suppressor T-cell effect. However, the data could also be interpreted as the result of a reduced number of stimulating B cells in the culture.

If one accepts that autologous B cells stimulate T cells, then the numerous reports that frozen leukemic blast cells sometimes stimulate autologous lymphocytes in remission (10-13) could be reinterpreted as not demonstrating the presence of a leukemic antigen as has been previously believed. If the leukemias were of B-cell type, (14) the autostimulation could well be of the same kind as that seen with lymphocytes from normal persons. Cultured lymphoblasts have also been shown to stimulate autologous lymphocytes (15, 16), and again most cultured cell lines are known to be B-cell lines (17). The rare cultured T-cell lymphoblast lines have been reported to be nonstimulating in MLC (18), which is consistent with the findings reported here.

Stimulation of lymphocytes by autologous tumor cells (19, 20) has been interpreted as stimulation by tumor antigens. Conceivably, contaminating B cells or macrophages could account for stimulation as shown here. Autologous
stimulation which has been considered to be the best "proof" of tumor antigens
may therefore not be sufficient by itself.

Summary

The background stimulation universally seen when lymphocytes are cultured
in vitro has been shown to be markedly lowered by reducing the proportion of B
lymphocytes. B-rich fractions of lymphocytes had extremely high background
stimulation. It is concluded that stimulation of T cells, probably by autologous B
cells, provides the most probable explanation for the findings described.

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