PRE-EMPTION OF HUMAN CELL-MEDIATED LYMPHOLYSIS
BY A SUPPRESSIVE MECHANISM ACTIVATED IN MIXED
LYMPHOCYTE CULTURES*

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The regulation of B-cell and T-cell immune responses has been extensively
examined and in the experimental animal appears to involve regulatory or
"suppressor" T cells (1-4). The limitations of in vitro experimentation have
made comparable study of nonpathological human suppression quite difficult
(5). We report here an in vitro method that generates and quantitates suppres-
sor activity in man after antigen-specific activation in mixed leukocyte culture
(MLC).

The one-way MLC induces both a proliferative response (6) and the generation
of cytotoxic T lymphocytes (CTLs) (7). Both of these responses are mediated by
antigen-specific T-cell subpopulations (8, 9) and have been correlated with
recognitive and destructive phases of allograft rejection. Recent reports have
examined the antigen reactivity of mouse (10, 11), rat (12), or human (13, 14)
lymphocytes obtained after proliferation in MLC. In all cases, after the primary
MLC proliferative peak, the recovered lymphocytes rapidly differentiate upon
re-exposure to the initial stimulating population, but do so only weakly when
exposed to a presumably noncross-reactive third-party stimulating population.

Velocity sedimentation separation studies have shown that the blast cells
produced in a primary MLC revert to small lymphocytes that rapidly differen-
tiate into proliferating and/or cytotoxic T lymphocytes upon restimulation with
the initial antigen (15). These findings demonstrate that positive selection for
the responding population in primary MLC does exist and may account for at
least part of the specificity of the secondary response. However, this positive
selection does not preclude possible involvement of a suppressor mechanism. In
fact we have detected suppressor activity in primary MLC sensitization cultures
at a time when the proliferation responsible for positive selection is not yet
significant, suggesting that suppression may be of overriding importance in the
specificity of MLC-activated secondary responses.

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TABLE I

Reciprocal CML Pre-emption

| CTLs                  | Cytotoxicity            |
|-----------------------|-------------------------|
|                       | Day 6                  | Day 8                  |
|                       | B targets K/T | C targets K/T | B targets K/T | C targets K/T |
|                       | Day 6 | Day 8 | Day 6 | Day 8 | Day 6 | Day 8 | Day 6 | Day 8 |
|                       | 30    | 30    | 30    | 30    | 30    | 30    |
|                       | %     | %     | %     | %     | %     | %     |
| 1 AB<sub>m</sub> -    | 54    | 33    | 14    | 2     | 33    | 12    | 5     | 3     |
| 2 AC<sub>m</sub> -    | 8     | 2     | 60    | 37    | 1     | 1     | 46    | 28    |
| 3 AB<sub>B</sub>C<sub>m</sub> - | 44    | 30    | 61    | 35    | 34    | 14    | 52    | 24    |
| 4 AB<sub>m</sub> -    | 50    | 38    | 29    | 18    | 43    | 23    | 11    | 6     |
| 5 AC<sub>m</sub> -    | 21    | 14    | 69    | 48    | 12    | 2     | 64    | 35    |
| 6 A C<sub>n</sub> -   | 6     | 4     | 28    | 16    | 9     | 2     | 65    | 45    |
| 7 A B<sub>n</sub> -   | 21    | 15    | 5     | 3     | 48    | 24    | 10    | 3     |
| 8 AA<sub>m</sub> -    | —     | —     | —     | —     | 7     | 2     | 70    | 44    |
| 9 AA<sub>n</sub> -    | —     | —     | —     | —     | 53    | 28    | 7     | 3     |
| 10 BC<sub>n</sub> -   | 1     | 1     | 57    | 30    | —     | —     | —     | —     |
| 11 CB<sub>n</sub> -   | 40    | 24    | —     | —     | —     | —     | —     | —     |

Each culture flask contained 9 x 10<sup>8</sup> responding cells and 12 x 10<sup>8</sup> stimulating cells added on day 0 or day 2. If two populations of stimulating cells were added, 6 x 10<sup>8</sup> of each were used. All CML combinations were tested on days 6 and 8 at killer to target ratios (K/T) of 30 and 6 to 1. Control value counts per minute for each target: Day 6 B Spontaneous Release (SR) = 156 Max = 1,079; Day 6 C SR = 198, Max = 852; Day 8 B SR = 168, Max = 1,498; Day 8 C SR = 163, Max = 1,105.

Materials and Methods

Methods used for generating CTLs and performing cell-mediated lympholysis (CML) assays in these studies have recently been described (16). All lymphocytes were Ficoll-Hypaque purified and obtained from healthy nontransfused volunteers. The protocols involved in these suppression studies required CML testing on two target populations that were minimally cross-reactive in the CML assay; however, this, relatively sensitive CML assay detects extensive cross-reactivity between most individuals examined. Therefore, many individuals were screened by prior CML testing to identify five different groups of three donors each. To simplify presentation of data, the responding cell donor in each group is designated A, and the other two B and C. These three individuals were in each case chosen so that CTLs from an AB<sub>m</sub> culture induced strong specific killing on B targets and low cross-killing on C targets; the CTLs from an AC<sub>m</sub> culture killed reciprocally.

Results

When fresh lymphocytes from individual A are simultaneously sensitized with mitomycin C-treated cells from B and C (B<sub>m</sub> and C<sub>m</sub>), cytotoxicity is developed against both B and C targets. CML blocking studies have shown that such cultures generate separate populations of CTLs, one reactive to antigens on B and the other reactive to C (16). Unlike simultaneous addition of B<sub>m</sub> and C<sub>m</sub>, the addition of C<sub>m</sub> to an ongoing AB<sub>m</sub> culture that was initiated 2 days earlier induces very little cytotoxicity on C, but allows the development of normal cytotoxicity on B targets. In this case the ongoing AB<sub>m</sub> culture "pre-empted" the cytotoxic response of the subpopulation of A cells able to recognize C.

An example of CML pre-emption is presented in Table I. In the standard day 6 CML assay, individual A demonstrated great specificity in distinguishing B target cells from C target cells (rows 1 and 2); however, even this low level of cross-killing was greater than the "autokilling" (rows 10 and 11). Rows 3 and 4 represent CTLs obtained from sensitization flasks that contained the same
number of A, Bm, and Cm cells, the only difference being that fresh Cm cells were added on day 2 in row 4, as opposed to on day 0 in row 3. These two populations killed B target cells to the same extent, as did the CTLs sensitized to Bm alone (row 1). Cytotoxicity on C targets by the ABm-Cm culture (row 3) was similar to that mediated by the ACm culture (row 2); both of these were much greater than that mediated by the ABm culture stimulated on day 2 with Cm (ABm-Cm, row 4). The cytotoxicity mediated on C targets by 30 x 10⁴ CTLs from this pre-empted culture (row 4) was 29%, less than that mediated by only 6 x 10⁴ CTLs in rows 2 and 3. Because peak CML activity is observed 6 days after allogeneic stimulation, it is essential to examine the cytotoxic activity of these cultures on day 8, the expected time of peak response to the stimulating cells added on day 2. Of greatest importance was the cytotoxicity mediated by the "pre-empted" mixture (row 4). Like rows 1 and 3, cytotoxicity on B targets dropped from day 6 to day 8. However, unlike the increased cytotoxicity directed at C targets in rows 6 and 8, the day 8 cytotoxicity on C targets by this pre-empted combination was as low as the cross-killing by ABm on the C targets (row 7). This indicated that the response of the A lymphocytes to the addition of Cm cells on day 2 was pre-empted by the ongoing response to Bm-stimulating cells. That the Cm cells obtained on day 2 were highly stimulatory was demonstrated by the cytotoxicity on C target cells in rows 6 and 8; the percent cytotoxicity was of comparable magnitude to that observed on day 6 using the standard CTLs stimulated on day 0 with Cm cells (rows 2 and 3).

The reciprocal combination and its appropriate controls also demonstrated that the ongoing response to Cm pre-empted the generation of CTLs to B target cells when Bm-stimulating cells were added on day 2 (row 5). This pre-emption is similar to preliminary data obtained in mouse (17).

In other experiments, pre-emption in man was observed by adding Cm to a 24-h ongoing ABm culture; the pre-emption effect increased slightly from day 1 to 3 (80–96% inhibition of cytotoxic potency). The observation of pre-emption before detectable MLC or CML reactivity and 4–5 days before their peaks would not be expected from a mechanism involving only positive selection for the responding (proliferating) population.

Direct evidence supporting a suppressive mechanism is presented in Table II. Fresh Cm-stimulating cells were added to ongoing 2 day ABm or AAm cultures with or without fresh A lymphocytes. The ABm cultures pre-empted the cytotoxic response to fresh Cm alone (row 5 compared to rows 4 and 6). If this pre-emption were merely selective in nature it would not be expected to influence fresh A lymphocytes from responding to Cm. However, the ongoing ABm culture (row 8), but not the AAm culture (row 7), markedly suppressed the expected development of cytotoxicity directed towards C (row 3) when fresh A plus Cm cells were added to them on day 2.

The combinations presented in the last four rows involved the addition of fresh A cells on day 2 and fresh Cm cells on day 3. Again, the ongoing ABm response initiated on day 0 suppressed the generation of CTLs directed against C. The observed suppressive effect required the responding ABm cells, since the cell-free supernate from a 2 day ABm culture did not suppress (row 12).

In other experiments, cells from a ABm sensitization flask were removed and
In Vitro Induced CML Suppression

CML reactions were performed with CTLs obtained from sensitization flasks to which responding and stimulating cells were added on days 0, 2, or 3. Each flask received a total of 9 x 10⁸ responding cells and 12 x 10⁸ stimulating cells. Control values for each target: B targets: SR = 179, Max = 1,441; C targets: SR = 379, Max = 1,904.

' Fresh A lymphocytes were cultured on day 2 in medium that had been cultured since day 0 in the absence of cells.
# Fresh A lymphocytes were cultured on day 2 in cell-free supernate from AB₄ culture established on day 0.

washed after 2 days of culture and added to fresh A plus C₄ cells; suppression similar to the above was caused by these cells, while the 2 day AB₄ supernate had no suppressing effect.

To determine where the suppressive mechanism was acting, cells from an ongoing 2-day AB₄ culture were added to an ongoing 2-day AC₄ culture. The cytotoxicity observed on day 6 was similar to that from a simultaneously stimulated AB₄C₄ culture, showing no suppressive effect. This suggests that the suppression mechanism demonstrated above involves inhibition of immune recognition or of the early steps in CTL differentiation.

Discussion

These experiments have demonstrated that a cell-dependent suppression of CTL activation is generated in human MLC. Several distinct methods of generating and detecting suppressor activity have recently been described (1–5, 18–22); aspects of this in vitro suppression in man appear to parallel certain qualities of in vitro induced murine suppression (23). However, more studies are required to determine the in vivo significance and the specific cellular mechanism of this suppression. Many complex models could be constructed to account for the phenomena, yet this seems unwarranted until more insight is provided. At present, two conclusions can be derived from these studies. Firstly, cell-mediated suppression of immune responses can be generated and studied in vitro using human lymphocytes responding to allogeneic cells. Secondly, the specificity of secondary responses to alloantigens after sensitization in MLC represents,
at least in part, a "pre-emption" of third-party responsiveness by this suppression mechanism.

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