TWO DISTINCT MECHANISMS REGULATE THE IN VIVO GENERATION OF CYTOTOXIC T CELLS*

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In this communication we explore the regulation of hapten-specific killing to both the trinitrophenol (TNP)- and azobenzenearsonate (ABA)-coupled cells. We present evidence indicating that Lyt-1+ T cells are required for the generation of hapten-specific CTL. This helper T cell requirement can be replaced by some factor(s) present in the supernatant of rat spleen cells stimulated with Con A (Con A-Sup). In addition, the generation of hapten-specific killing is susceptible to tolerization. At least two distinct mechanisms of unresponsiveness appear to be operative. Regulation by antigen-specific T cells appears to function at the level of helper T cell. Furthermore, a second nontransferable state of unresponsiveness is found. The significance of these findings will be discussed in terms of cellular regulation of generation of CTL in mice.

Materials and Methods

Mice. A/J (H-2k), BALB/c (H-2d) mice were obtained from The Jackson Laboratory, Bar Harbor, ME. All the animals used in this study were maintained in accordance with the guidelines of the Committee on Animals of the Harvard Medical School and those prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW publication [NIH] 78-23, revised 1978).

Preparation of Antigen and Antigen-coupled Cells. The ABA coupling of spleen cells has been described in detail elsewhere (1). TNP-conjugated spleen cells were prepared as described (2).

Induction of Immunological Unresponsiveness and Suppressor T Cells

Induction of immunological unresponsiveness. To induce unresponsiveness, mice were injected intravenously with $5 \times 10^7$ ABA-SC or TNP-SC. 7 d before priming, mice were injected with $3 \times 10^7$ ABA-SC subcutaneously.

Assay of suppressor T cells. $5 \times 10^7$ spleen cells from mice treated as above were transferred intravenously into another group of naive recipients that were immunized subcutaneously with $3 \times 10^7$ ABC-SC or TNP-SC. 5-7 d later, their spleens were removed and restimulated in vitro with hapten-SC for the induction of CTL.

In vitro culture. The culture conditions used are described in detail elsewhere (1-3). Briefly, $7 \times 10^6$ spleen cells from primed animals were co-cultured with $6 \times 10^6$ ABA- or TNP-coupled x-irradiated (1,400 rad) spleen cells in a volume of 2 ml for 5 d.

Chromium release assay. Cytotoxicity was calculated on the basis of the formula: percent specific release = $e - c)/(Ft - c) \times 100\%$, where c is the spontaneous release obtained by incubating target cells with normal spleen cells in the 4-h assay. Ft value was calculated by four freeze-thaw cycles. Spontaneous release of tumor cells usually ranged from 8-16% in the 4-h

* Supported in part by grants NIAID 16701 and AI 14732 and training grant ZT32 CA-09130-06 from the National Institutes of Health.
‡ Supported by Mitsubishi Chemical Co., Tokyo, Japan.
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The assays were performed in duplicate. All cultures were assayed on both modified and unmodified targets, and the background lysis on unmodified targets was substrated. All the experiments have been done at least three times, with comparable results.

Rat Con A Supernatant. The preparation of Con A-stimulated rat spleen cell culture supernatant has been described in detail (4). Supernatants used in this study were all from one lot, which gives 50% maximum cpm of the IL-2-dependent L2.2 cell line (4) at a dilution of 1:50. Control supernatants consist only of alpha-methyl mannoside in identical media. All dilutions shown are final concentrations added at the beginning of culture.

Serum Treatment. Monoclonal anti-Ly-1.2 antibodies were used at 1:200 and were kindly provided by Dr. F. W. Shen (Memorial Sloan-Kettering Cancer Institute, New York). \(5 \times 10^7\) spleen cells were incubated for 45 min at room temperature with 1 ml of diluted anti-Ly-1.2 antibody, washed twice, and then incubated with 1 ml of 1:7 dilution of Low Tox rabbit complement (Accurate Chemical and Scientific Corp., Westbury, NY) for 30 min at 37°C.

Results and Discussion

In our previous report, we found that in vivo priming followed by in vitro restimulation was required for generation of hapten-specific CTL and that adoptive transfer of suppressor T cells (Ts) induced by intravenous injection of hapten-conjugated spleen cells (TNP-SC) can limit the in vivo priming of cytotoxic T cells (3). This requirement for in vivo priming has been suggested to be at least in part necessary for the activation of a helper T cell subpopulation, which provides a differentiation signal for the activation of cytotoxic T cells (1, 5).

To further investigate the role of helper T cells in the generation of ABA-specific cytotoxic T cells in vitro, we examined the effect of treating the responder cells with a monoclonal anti-Ly-1.2+ antibody plus complement to eliminate Ly-1.2+-bearing helper T cells. As shown in Table I, treatment of responder cells with anti-Ly-1.2+ antibodies plus complement completely abrogated their ability to generate CTL.

To further clarify whether the elimination of ABA-specific CTL response by anti-Ly-1.2+ plus complement treatment is due to the removal of helper T cells or elimination of Ly-1.2,3+ cytotoxic T cell precursors (6), we investigated the effect of adding Con A-Sup in vitro. Our results shown in Table I clearly demonstrated that in vitro addition of Con A-Sup can restore the response of aLy-1.2+ plus complement-treated responder cells. Thus, the CTL is not appreciably altered by the aLy-1.2+ treatment, but requires a Ly-1.2+ cell that can be replaced by factors present in Con A-Sup. The mechanisms and the molecules in the Con A-Sup that are responsible for providing the necessary signals for the induction of CTL have not been addressed in our experiment. The molecule responsible for the helper activity might be IL-2, which is present in the Con A-Sup (7), as purified IL-2 has been reported able to provide nonspecific helper signals required for the activation of CTL (8). Based on the above experiments, we investigated the mode of action of hapten-specific Ts. Because two cellular components are required for the generation of effector CTL, Ts might work either by inhibiting the helper T cell or by limiting the CTL. In addition, because we were unable to demonstrate any Ts activity in vitro mixing experiments (unpublished results), we postulated that Ts are functioning only during in vivo priming. If the Ts works primarily at the level of helper T cells, we would expect CTL precursors to be present in a suppressed animal. To address this issue, an exogenous source of helper signals was added to the in vitro restimulation of a suppressed culture.

The result of such an experiment is shown in Table II. TNP-specific Ts induced in BALB/c mice when transferred into naive recipients will significantly inhibit the in
**Table I**

**Effect of Anti-LYT-1.2 plus Complement Treatment on the Generation of ABA-specific CTL**

| Responder cells* | Responder cell treatment | In vitro addition of Con A-Sup | Percent specific lysis‡ |
|------------------|--------------------------|------------------|----------------------|
|                  |                          | Effector-to-target ratio |                      |
|                  |                          | 80:1 27:1 20:1 9:1   |                      |
| ABA-SC (s.c.)    | C'                       | 55 32 15 20         |                      |
| ABA-SC (s.c.)    | Anti-Ly-1.2 + C'         | 0 0 0 0             |                      |
| ABA-SC (s.c.)    | Anti-Ly-1.2 + C' + (1:5) | 70 58 42 30         |                      |
| ABA-SC (s.c.)    | Anti-Ly-1.2 + C' + (1:10)| 60 59 30            |                      |

* Responder cells were obtained from the spleen of A/J mice that were injected with 3 × 10⁷ ABA-SC subcutaneously 7 d earlier. 7 million responder cells were co-cultured with 6 × 10⁶ ABA-SC (A/J) for 3 d.
‡ After 5 d, cytolytic activity was assayed on ABA-modified P815 tumor cells. In this experiment spontaneous release was 16.6%.

**Table II**

**Reversal of TNP Suppressor T Cell Activity by In Vitro Addition of Con A Supernatants**

| Pretreatment of responder cell donors* | In vitro addition of Con A supernatants | Percent specific lysis, effector/target ratio‡ |
|--------------------------------------|----------------------------------------|---------------------------------------------|
|                                      |                                        | 10:1 33:1 1:1                                |
| (A) TNP-SC (s.c.)                    | —                                      | 64.2 66.2 58.4                               |
| (B) TNP-SC (s.c.) + 5 × 10⁷ Ts₁       | —                                      | 64.3 65.5 62.6                               |
| (C) TNP-SC (s.c.) + 5 × 10⁷ Ts₁       | + (1:10)                               | 61.5 31.5 48.7                               |
| (D) TNP-SC (s.c.) + 5 × 10⁷ Ts₁       | + (1:30)                               | 38.6 31.5 19.7                               |
| (E) TNP-SC (s.c.) + 5 × 10⁷ Ts₁       | Control supernatants                   | 80:1 26:1 9:1                                |

* Responder cell donors were either immunized with 3 × 10⁷ antigen-coupled spleen cells (s.c.) with or without concomitant 5 × 10⁷ suppressor T cells (s.v.), as described in Materials and Methods.
‡ Cytolytic activity was assayed on TNP-modified P815 cells in the TNP experiment or on ABA-modified P815 in the ABA experiment. Spontaneous release in the TNP was 12.9% and in the ABA, 16.6%.

The results are presented in the lower one-half of Table II. ABA-specific Ts₁, when transferred into naive recipients, inhibits the in vivo priming of ABA-specific CTL. Similar to the observation in the TNP system, suppression in the ABA system can also be reversed by in vitro addition of Con A-Sup. These results, in some respects, mimic the aLy-1.2* plus complement experiment (Table I) and thus allow us to suggest that Ts might inhibit the generation of CTL by inhibiting the helper T cells. Our results are consistent with the hypothesis that the Con A-Sup reversed suppression by providing a helper signal in the same way that it provides the necessary signal in the aLy-1.2*–depleted cultures. This observation is in some respects similar to the earlier findings of Butler et al. (9). The mechanisms whereby Ts regulates helper T
Table III

Two Distinct Mechanisms Regulate the In Vivo Induction of TNP-specific Cytotoxic T Cells

| Pretreatment of responder cell donors* | In vitro addition of Con A supernatants | Percent specific lysis |
|--------------------------------------|----------------------------------------|-----------------------|
|                                       | Control supernatants                    | 90:1 30:1 10:1        |
| Day −7 Day 0                          |                                        |                       |
| (A) − TNP-SC (s.c.)                   | —                                      | 79.8 77.5 58.5        |
| (B) − TNP-SC (s.c.) + Tsl             | + (1:20)                               | 48.2 20.4 9.1         |
| (C) − TNP-SC (s.c.) + Tsl + (1:20)    | + (1:30)                               | 17.6 7 5              |
| (D) − TNP-SC (s.c.) + Tsl + (1:30)    | Control supernatants                    | 0 1 0                 |
| (E) − TNP-SC (i.v.)                   | TNP-SC (s.c.)                          | 0 0 0                 |
| (F) TNP-SC (i.v.)                     | TNP-SC (s.c.)                          | 0 0 0                 |
| (G) TNP-SC (i.v.) + Tsl               | + (1:20)                               | 0 0 0                 |
| (H) TNP-SC (i.v.) + Tsl + (1:30)      | Control supernatants                    | 0 0 0                 |
| (I) TNP-SC (i.v.)                     | Control supernatants                    | 100:1 33:1 11:1       |
| (A) − ABA-SC (s.c.)                   | —                                      | 31 20.9 5.8           |
| (B) − ABA-SC (s.c.) + Tsl             | —                                      | 0 3 1                 |
| (C) − ABA-SC (s.c.) + Tsl + (1:10)    | + (1:10)                               | 35.2 15 5.6           |
| (D) − ABA-SC (i.v.)                   | ABA-SC (s.c.)                          | 1 3 5                 |
| (E) ABA-SC (i.v.)                     | ABA-SC (s.c.)                          | 6.8 4 5               |

* Responder cells were prepared as described in the text.
† Cytolytic T cell activity was assayed on TNP-modified P815 cells in the TNP experiment and ABA-modified YAC tumor cells in the ABA experiment. Spontaneous release: TNP, 14%; ABA 33%.

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added a three times higher concentration of Con A-stimulated culture supernatant, no significant increase in CTL activity was observed. Another possibility is that there are additional subpopulations of Ts in the tolerized animals that work directly on CTL or their precursors and, thus, result in a nonreversible defect. In addition, it is possible that intravenous injection of hapten-conjugated spleen cells not only induces Ts, but also results in a direct blockade of CTL precursors. A dual mechanism of immunological unresponsiveness involving both Ts and receptor blockade has been shown to exist in the regulation of contact sensitivity to DNFB in mice (14).

It is not known why unresponsiveness induced by water-soluble TNBS can be reversed by Con A-Sup (9), whereas unresponsiveness induced by TNP-SC failed to do so. However, it has been shown (14) in the regulation of the contact sensitivity system that intravenous injection of DNBS and DNP-SC represents two very different tolerogenic regimens. In addition, reports from other laboratories (15-17) were unable to induce immunologic tolerance in vivo using TNP-SC, but the experimental protocol these investigators used is quite different from ours. In our experimental design, after intravenous tolerization, there is an additional in vivo priming before in vitro restimulation.

The inability of Con A-Sup to reverse unresponsiveness has certain implications concerning the mechanism of activation of CTL. It has been proposed recently (5) that two helper T cells might be required for the activation of CTL. Unprimed CTL precursors in vivo might require a differentiation signal from one helper T cell and a proliferation signal (probably mediated by IL-2) from a second set of helper T cells in the presence of antigen to generate CTL. In the absence of the first signal, unprimed CTL precursors were unable to accept the IL-2 signal. Therefore, it is possible that intravenous injection of hapten-conjugated spleen cells might have inactivated the first helper T cell that is required for the delivery of the first signal and, thus, resulted in the failure to generate any CTL even in the presence of exogenous helper signals. On the other hand, suppressor T cells, when adoptively transferred to a recipient, might allow the activation of the first helper T cells but inhibit the activity of second set of helper T cells. Experiments are now in progress to address these issues.

Summary

Treatment of responder cells with monoclonal anti-Ly-1,2 antibodies plus complement in vitro completely eliminated their ability to generate azobenzenearsenate (ABA)-specific cytolytic T lymphocytes (CTL). However, addition of the concanavalin A-stimulated supernatants of rat spleen cells (Con A-Sup) can fully reconstitute the response. Therefore, Lyt-1,2-bearing T cells are required for the generation of ABA-specific CTL, and such requirement can be replaced by factors present in the Con A-Sup. Suppressor T cells (Ts), when adoptively transferred into naive recipients, will inhibit the in vivo priming of CTL. This inhibition can also be reversed by in vitro addition of Con A-Sup. Furthermore, mice serving as donors of Ts also show profound unresponsiveness when primed and restimulated in vitro. In contrast to the Ts-mediated inhibition, in vitro addition of Con A-Sup was unable to abolish the unresponsiveness observed in these cultures. Thus, we identified two unresponsive states in a hapten-specific killing system that differ in their ability to be reconstituted by Con A-Sup.

Received for publication 17 May 1982 and in revised form 22 June 1982.
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