Brief Definitive Report

GENERATION OF ANTIGEN RECEPTOR–SPECIFIC SUPPRESSOR T CELL CLONES IN MAN

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T cells that use noncytolytic mechanisms to downregulate the immune response (i.e., suppressor cells) are thought to play an essential role in controlling reactivity to foreign antigens and inducing tolerance to self antigens. Despite more than a decade of intense study, however, our knowledge of the mechanisms whereby suppressor T (Ts) cells are activated is incomplete. For example, while cytotoxic and helper T cells use well-characterized receptors to recognize antigen and distinguish self from nonself (1), the nature of receptor-ligand interaction that mediates activation of Ts cells is not yet known. One suppressor system, which has been studied (2) extensively in mice, postulates direct interaction between Ts cells and antigen in a manner analogous to that described for B cells. Recently (3–7) we have described in humans a form of Ts cell which is activated by direct interaction with antigen receptors on antigen-primed inducer T cells. Like the antigen-specific Ts cells described in mice, the human Ts cell suppresses immune responses to the priming antigen but not irrelevant antigens.

Although occasional reports of long-term antigen-specific Ts cell lines have appeared (8–10), a reproducible method for propagating Ts cells as functional clones has not yet been developed. The inability to generate Ts clones has undoubtedly contributed to the suppressor cell enigma, since detailed analysis of functionally important cell surface molecules is difficult without a homogeneous, functionally intact cell source. In the present study, we show that CD4⁺ inducer blasts can be used in combination with IL-2-containing medium to reproducibly propagate CD8⁺ suppressor T cell clones, which inhibit immunity in an antigen receptor–specific, genetically restricted manner.

Materials and Methods

Monoclonal Antibodies. Anti-Leu-2, anti-Leu-3, anti-Leu-4 (CD3), anti-HLA-DR, anti-Leu-10 (HLA-DQ), and anti-Leu-15 (CD11) antibodies were obtained from Becton Dickinson Monoclonal Center, Mountain View, CA. Antibody 9.3 was kindly provided by Dr. J. A. Hansen, Hutchinson Cancer Research Center, Seattle, WA. Analysis of surface phenotype with these antibodies was performed on an Ortho System 50H Cytofluorograf as described (6).

Preparation of T and Non-T Cells. Peripheral blood mononuclear leukocytes (MNC) from healthy volunteers were separated into T and non-T cells with a sheep erythrocyte

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rosetting technique (3). T cells were separated into CD8\(^+\) (Leu-2\(^+\)) and CD4\(^+\) (Leu-3\(^+\)) cells by panning with mAbs (3) and suspended in complete medium (CM). CM consisted of Iscove’s modified Dulbecco’s medium (IMDM) (Gibco, Grand Island, NY) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 \(\mu\)g/ml streptomycin, and 10% FCS.

**Generation of Inducer T Cell Lines.** CD4\(^+\) T cells were activated in MLR with irradiated (8,000 rad) EBV-transformed B cells. The resultant CD4\(^+\) lymphoblasts were isolated on Ficoll-Hypaque gradients and propagated in microwells containing CM supplemented with IL-2-containing supernatant in the presence of irradiated (8,000 rad) allogeneic stimulator cells. IL-2-containing supernatant was generated from activated MNC as described (5), and 5 U IL-2 per milliliter was added every 3–4 d, alternating with irradiated stimulator cells (5 \(\times\) 10\(^5\) cells/ml).

**Generation of Suppressor T Cell Clones.** Alloantigen-primed CD4\(^+\) lymphoblasts were recovered on Ficoll-Hypaque gradients, irradiated (3,000 rad), and used to stimulate fresh autologous CD8\(^+\) T cells. 7 d later, CD8\(^+\) T cells were recovered on Ficoll-Hypaque gradients, and residual CD4\(^+\) inducer cells were removed by panning with anti-CD4. Activated CD8\(^+\) cells were cloned at 1 cell/well in round-bottomed microtiter wells in 200 \(\mu\)l CM supplemented with IL-2-containing supernatant and 10\(^5\) irradiated autologous CD4\(^+\) inducer blasts.

**Assay for Suppression.** CD8\(^+\) clones were irradiated (2,000 rad) and added in graded numbers to indicator cultures consisting of 5 \(\times\) 10\(^4\) fresh autologous CD4\(^+\) T cells and 10\(^5\) irradiated (8,000 rad) allogeneic stimulator cells in a final volume of 200 \(\mu\)l. Quadruplicate microwells were pulsed with \(^3\)H thymidine (6.7 Ci/mM, 1 \(\mu\)Ci/well; New England Nuclear, Boston, MA) on day 6, and the cells were harvested 16 h later. Percent suppression was calculated as: 100 \(\times\) [1 - (\(\Delta\) cpm in cultures with cloned CD8\(^+\) T cells as regulators)/\(\Delta\) cpm in control cultures)]. Where \(\Delta\) cpm represents cpm value obtained after subtracting background counts in unstimulated cultures.

**Results**

CD8\(^+\) T cells from three unrelated individuals were incubated for 7 d with autologous alloactivated CD4\(^+\) cells, after which activated CD8\(^+\) Ts cells were isolated and cloned in the presence of irradiated autologous alloreactive CD4\(^+\) cells and IL-2-containing medium. Of 1,800 wells initially seeded at 1 cell/well, 39 (13 from KC, 6 from CB, and 20 from ST) could be expanded. After expansion for 30 d, the surface phenotypes of the cloned cells were determined, and remarkably, each of the 39 clones was CD3\(^+\), CD4\(^-\), CD8\(^+\), CD11\(^-\), 9.3\(^-\), DR\(^+\), DQ\(^+\). This phenotype was maintained as long as the clones were grown (up to 5 mo).

The cloned cells were added in graded numbers to indicator MLR cultures consisting of fresh autologous CD4\(^+\) cells and the original allogeneic stimulus. As shown in Fig. 1, suppressive clones were obtained from all three subjects. Although the potency of suppression on a per cell basis varied among these clones, the magnitude of suppression was in all cases directly proportional to the number of Ts cloned cells in the indicator MLR cultures. The most potent clones, such as CB1, CB5, KC2, ST5, and ST6 inhibited fresh MLRs by >80% at ratios of Ts cells to fresh T cells of 1:2 and 1:4. Other suppressive clones such as KC1 and KC3 were less potent, while several clones (not shown) were not suppressive over a wide concentration range. The CD8\(^+\) clones with suppressive properties also blocked the response of autologous CD4\(^+\) feeder cells to the original allogeneic stimulator line (data not shown).

To explore the specificity of this suppressive effect, Ts clones were added to MLRs consisting of fresh autologous CD4\(^+\) cells and allogeneic stimulator cells.
Suppression of MLR by cloned CD8+ cells. Graded numbers of cloned CD8+ T cells from KC, CB, and ST were cultured with 5 × 10^6 fresh autologous CD4+ cells and 10^4 allogeneic stimulator cells. In each case, the allogeneic stimulators were those that had been used to activate CD4+ inducer cells which were, in turn, used to stimulate autologous CD8+ cells. [3H]Thymidine incorporation in these cultures was measured on day 7, and percent suppression was calculated as described in Materials and Methods. SEM of replicate cultures was <10% in all cases.

The results (Table I) show that the suppressor effect of CD8+ Ts clones was specific for the original allogeneic stimulus. For example, ST clones, which were grown on HLA-DR5-reactive CD4+ T cells, inhibited the response of fresh ST CD4+ cells to a DR5+ B cell line, but not to a DRw6+ B cell line. Similarly, KC and CB clones, which were propagated on HLA-DRw6-reactive CD4+ T cells, suppressed the response of fresh, autologous CD4+ cells to the ARENT B cell line (HLA-A2,2; B38,39; DRw6,6), but not to third party...
stimulators such as the B cell lines MSAB (HLA-A1,2; Bw57,57; DR7,7) and BRG (HLA-A1,1; B8,8; DR3,3). In studies using a more extensive panel of stimulator cells, the CB1 clone suppressed the response to all DRw6 lines, including Daudi, a B cell line that expresses DRw6 molecules but no HLA-A or -B molecules, but it failed to inhibit the response to a variety of lines lacking DRw6, regardless of their HLA-A and -B phenotype (data not shown). This specificity of suppression raised the possibility that the suppressive effect was due to cell-mediated lymphotoysis of either the autologous CD4+ responders or the original allogeneic stimulator cells. However, in 4 h cytotoxicity assays, none of the suppressive clones caused detectible lysis of either target at E/T ratios as high as 40:1 (data not shown).

Additional experiments were undertaken to determine whether the Ts clones are genetically restricted in their inhibitory effects. Table II shows that the HLA-DRw6-specific Ts clones CB4 and CB5 failed to suppress the response of an individual who shared HLA-DR but not HLA-A or -B alloantigens with the suppressor donor. On the other hand, the responses of the suppressor donor and an individual who shared HLA-A and -B but not -DR with the suppressor donor were suppressed by both Ts clones. These results suggest that the interaction between suppressor cells and their target (CD4+ responder T cells) is genetically restricted, most likely to class I MHC gene products or to products of genes linked to class I MHC.

| Donor of Ts clone | Initials | HLA | Ts clone added | Original stimulus | Third party stimulus |
|-------------------|----------|-----|----------------|-------------------|---------------------|
|                   |          |     |                | HLA              | Response (cpm ± SEM) | HLA | Response (cpm ± SEM) |
| ST                | A24,(-)  | No Ts | A24,30      | 24,268 ± 5,645  | A2,2      | 24,399 ± 2,722  |
| B51,(-)           | ST6      |      | B18,Bw35    | 22,55 ± 458*    | B8,8      | 28,188 ± 5,036  |
| DR5,4             |          |      | DR5,5       |                  |          |                  |
| KC                | A11,24   | No Ts | A22         | 38,887 ± 1,637  | A1,2      | 42,717 ± 5,315  |
| B27,44            | ST6      |      | B38,39      |                  |          |                  |
| DR5,4             |          |      | DR6,6       | 1,141 ± 189*    | DR7,7     | 37,646 ± 2,097  |
| RB                | A1,2     | No Ts | A22         | 36,795 ± 1,302  | DR7,7     | 37,646 ± 2,097  |
|                 | B7,27    |      | B38,39      |                  |          |                  |
| DR5,4             |          |      | DR6,6       | 16,479 ± 5,642* | DR3,3     | 119,587 ± 9,486 |

5 x 10^6 fresh CD4+ T cells were stimulated with 10^6 EBV-transformed allogeneic B cells in the presence or absence of 2.5 x 10^5 cloned Ts cells.
* Suppression significant at p < 0.001.

| Responder CD4+ cells from: | HLA phenotype of responder | [\(^3\)H]Thymidine incorporation (cpm ± SEM) by CD4+ cells in presence of DRw6* Ts clone |
|---------------------------|-----------------------------|----------------------------------------------------------------------------------|
| CB                        | 1,2                         | None                                                                             |
| CB4                       | A2                         | 115,079 ± 6,625                                                                   |
| CB5                       | B2                         | 29,676 ± 2,863*                                                                  |
| CB                        | DR2,4                       | 30,062 ± 6,062*                                                                  |
| CB                        | DR5,4                       | 48,227 ± 5,781*                                                                  |
| CB5                       | DR6,6                       | 94,868 ± 114*                                                                    |

2.5 x 10^6 cells from the HLA-DRw6-specific Ts clones, CB4 and CB5, were examined for their ability to suppress the response of 5 x 10^4 CD4+ cells to the DRw6* B cell line, ARENT.
* Suppression significant at p < 0.001.
Discussion

In these studies, T cell clones that suppress the immune response in an antigen-specific manner have been generated reproducibly and maintained in continuous culture for periods as long as 5 mo. The method used to generate these clones was based on the earlier observation that fresh CD8+ T cells, when cultured for 1 wk with autologous antigen-primed CD4+ cells, developed into Ts cells with specificity for the initial inducer cell stimulus, either alloantigen (3) or soluble antigen (4). Since the clones described here and the bulk cultures described earlier are indistinguishable with respect to surface phenotype (CD3+, CD4-, CD8+, CD11-, 9.3-), target (CD4+ helper/inducer T cell), and genetic restriction (class I MHC), it seems reasonable to conclude that the clones are representative of the cell type responsible for the earlier observations.

The basis for the exquisite specificity of suppression presumably resides in the fact that the Ts cells recognize only those CD4+ cells that express a receptor for the original antigenic stimulus. Although the precise mechanism of suppression remains to be defined, Ts cells appear to exert their inhibitory effect by a noncytolytic, class I MHC-restricted mechanism. Previous studies (2) of antigen-specific Ts cells in mice suggested that suppression is genetically restricted by products of H-2 I region genes. However, studies of the type described here, using sequentially activated T cell subpopulations, have not been carried out with murine systems. In addition, in at least one murine system, an apparent I region-restricted function of antigen-specific Ts cells may not be related to the recognition of I region products on accessory cells, but rather to the MHC-restricted interaction between helper/inducer T cells and antigen presented on accessory cells (11).

Classical Ts cells of the type described in mice (12) have proven difficult to propagate under conditions that have worked well for helper and cytolytic T cells. There are many possible explanations for this failure, including a potential need for growth and differentiation factors distinct from those present in traditional conditioned media, and the possibility that such Ts cells do not respond to antigen but to antigen-reactive inducer T cells. It is noteworthy that the circuit of interactive murine T cells postulated to generate antigen-specific, MHC class II-restricted suppression is remarkably similar to that which, in our experience, generates antigen receptor-specific, MHC class I-restricted suppression. Thus, both systems are initiated by an MHC class II-restricted interaction between antigen/MHC and a T cell of helper/inducer phenotype (murine Ts1), which in turn activates a T cell of suppressor phenotype (Ts2) (2). In our view, the evidence that murine antigen-specific Ts cells are either activated by antigen or function by binding antigen is inconclusive. Regardless of the potential interaction between some types of Ts cells and antigen, since the conditions described here result in the reproducible propagation of at least one type of Ts cell, similar conditions could be used in efforts to generate and study other types of suppressor T cells.

Summary

We have shown previously that CD8+ T cells proliferate upon exposure to autologous, antigen primed CD4+ T cells, and suppress the response of fresh T cells to the priming antigen but not irrelevant antigens. The stimulus and target
of suppression in this system appears to be the antigen receptor on the surface of CD4+ cells, rather than the nominal antigen. In the current study, alloantigen primed CD4+ inducer cells and IL-2-containing medium were used to generate clones of suppressor cells from several individuals. The clones inhibited the response of fresh autologous T cells only to the original allogeneic stimulator cell and to stimulator cells that shared HLA-DR antigens with the priming cell. The clones were also genetically restricted, since they inhibited the response of HLA-A,B-compatible but not HLA-A,B-incompatible individuals. The availability of a method for reproducibly generating antigen receptor-specific suppressor T cell clones in vitro should make it possible to clarify the mechanism, whereby such cells are activated and exert their suppressive effect.

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