SUPPRESSIVE MECHANISMS IN ALLOANTIGEN-INDUCED T CELL RESPONSES*

By MARGARET BECKWITH and SUSAN RICH

From The Department of Microbiology and Immunology, Baylor College of Medicine, Houston, Texas 77030

Alloantigen-induced T cell proliferation and cytolytic T cell development in the mixed lymphocyte response (MLR)[1] involves an interaction of both augmenting and suppressive pathways (1–7). We have previously characterized a soluble suppressor factor, designated MLR-TsF, which is produced by alloantigen-primed, Ly-2^+ splenic T cells (7) and suppresses the mixed lymphocyte response in a genetically restricted manner (4–6). In initial studies to identify possible sites of MLR-TsF activity and define its mechanism of suppression, we determined that MLR-TsF exerts a component of its suppressive effect through induction of an Ly-1^+2^+ second-order suppressor T cell, termed Ts2 (7). Similar multicellular suppressive pathways involving several distinct Ts subsets and soluble inhibitory factors have been demonstrated for a variety of antigenic systems (8–12). However, despite the demonstration of at least three intermediate steps in suppression, in most systems the endpoint mechanism of suppression remains unclear.

Attempts to define the molecular requirements for T cell clonal expansion and terminal differentiation have recently led to identification of growth factors essential to T cell proliferation and cytolytic T cell development (13–15). Following antigen activation and an interaction with the macrophage produced interleukin, IL-1, T cells release IL-2 which then binds to high affinity receptors on activated T cells, thus driving their proliferation (14). This direct and obligate relationship between T cell proliferation and IL-2 suggested that interference with the interleukin cascade, during the processes of IL-2 production, receptor expression, binding, or utilization, might represent a general immunosuppressive mechanism responsible for the final inhibition observed in many of the diverse suppressive systems studied (15). We therefore investigated the possibility that inhibition of the mixed lymphocyte response might occur through MLR-TsF interference with IL-2 production or activity either directly, or as an endpoint

** Supported by U.S. Public Health Service Research Grant AI 13810. Computational assistance was provided by the Clinfo project, funded by the Division of Research Resources of the National Institutes of Health (NIH) under grant number RR-00350.

[1] *Abbreviations used in this paper:* CAS, IL-2 containing supernatant from Con A-activated murine splenocytes; Con A, concanavalin A; CTL, cytolytic T lymphocyte; HBSS, Hanks' balanced salt solution; IL-1, interleukin 1; IL-2, interleukin 2; MEM-2%, Eagle's minimal essential medium containing 2% fetal calf serum; MLR, mixed lymphocyte response; MLR-TsF, T cell factor suppressive of mixed lymphocyte responses; PBS, phosphate-buffered saline; PMA, phorbol myristic acetate; TdR, [^{3}H]thymidine; Ts, T suppressor cells; Ts2, second order T suppressor cells induced by MLR-TsF.

process of Ts2-mediated suppression.

We report here that the process of MLR-TsF mediated suppression appears to be comprised of two distinct activities. The first derives from a direct MLR-TsF interference with IL-2–driven proliferation of responder T cells and involves inhibition of events occurring subsequent to the IL-2 receptor interaction, rather than limitation of IL-2 production or decreased expression or binding capacity of IL-2 receptors. This suppressive activity is clearly separable from the previously described second-order suppressor cell induction (7), and in this paper we demonstrate that, although IL-2 may be required for Ts2 expansion, suppression is not predominantly a result of competition for or decreased production of IL-2. These results imply that the terminal event associated with Ts2 suppression of alloantigen-activated T cells may also involve later events in the IL-2–dependent lymphokine cascade.

Materials and Methods

Mice. BALB/c mice were obtained from the Department of Cell Biology, Baylor College of Medicine. C57BL/6 (B6) mice were purchased from The Jackson Laboratory, Bar Harbor, ME. All experiments utilized 6–10-wk old male mice.

Preparation of Suppressive Supernatants. Supernatants were prepared as described previously (4). BALB/c mice were immunized in the hind footpads with $30–50 \times 10^6$ B6 spleen cells. 4 d later primed BALB/c spleen cells were enriched for viable cells by hypotonic lysis and filtration through cotton-plugged columns, mixed with equal numbers of irradiated (1,500 rads) B6 stimulator cells, and cultured for 24 h at a total concentration of 20×10^6 cells/ml in supplemented Eagle's minimal essential medium (MEM) containing 2% heat-inactivated fetal calf serum (FCS) (Gibco Laboratories, Grand Island, NY). Supernatants were harvested and stored at −70°C for no longer than 2 wk before use. All suppressive supernatants used in this study were generated using B6-primed BALB/c responders and B6 stimulators, and are designated MLR-TsF.

IL-2 Production. Two sources of IL-2 were used. B6-derived EL4 thymoma cells of a cloned subline developed by Dr. J. Farrar (NIH) and obtained from Dr. E. Vitetta, University of Texas Health Sciences Center (UTHSC), Dallas, TX were resuspended to 1×10^6/ml in supplemented RPMI-1640 (MA Bioproducts, Walkersville, MD) containing 1% FCS and 12.5 ng/ml phorbol myristic acetate (PMA) (Sigma Chemical Co., St. Louis, MO). Supernatants were harvested after a 24-h incubation, and the IL-2–containing fraction was isolated by a 50–85% saturated ammonium sulfate precipitation. The precipitate was dissolved and dialyzed extensively against phosphate-buffered saline (PBS) before final dialysis into Hepes-buffered Hanks' balanced salt solution (HBSS) and storage at −20°C (16). Alternatively, a lectin-free, Con A supernatant (CAS) was prepared by incubating mouse splenocytes in supplemented RPMI-1640 containing 2% FCS and 10 μg/ml Con A (Sigma Chemical Co.) for 2 h at 37°C in an atmosphere of 5% CO_2. The cells were then centrifuged, washed, and resuspended in supplemented RPMI-1640 containing 10% FCS and incubated for an additional 20 h (17). Supernatants were harvested, filtered, and stored at −20°C until use. EL4 supernatants generally contained approximately 50–100 times more activity than the CAS when compared in the HT2 assay (see below). All experiments were done using both sources of IL-2 with no qualitative difference in results.

Generation of Ts2 Regulator Cells. Regulator cells were prepared as described previously (7). Briefly, 40×10^6 unprimed BALB/c spleen cells were enriched for viable cells, and cultured in a total volume of 4.0 ml containing 2.0 ml of supplemented MEM with 10% FCS (MEM-10%) and 2.0 ml of control medium (MEM-2%) or suppressive supernatants. The cultures were established in 60-mm petri dishes (Falcon 3002) and incubated at 37°C on a slowly rocking platform in an atmosphere of 10% CO_2, 83% N_2, and 7% O_2. After 48 h, cells were harvested, washed three times in HBSS, irradiated (1,500 rads), and

resuspended to 5×10^6/ml in MEM-10% before addition to the MLR assay.

MLR Assay. Mixed lymphocyte responses were established as described previously (4–7) with minor modifications. BALB/c responder and irradiated BALB/c or B6 stimulator cells were resuspended in MEM-10%, mixed in a 1:1 ratio, and plated in 96-well flatbottom microtiter plates in 0.1 ml volume. MLR-TsF was added to cultures containing 1×10^6 each responders and stimulators per well. Control and Ts2 regulator cells, prepared as described above, were added to MLR containing 5×10^5 each responder and stimulator cells. In some experiments 50 μl of an IL-2–containing supernatant was added to MLR in conjunction with MLR-TsF or regulator cells, increasing the total volume to 0.25 ml/well. MLR cultures were incubated at 37°C in an atmosphere of 10% CO_2, 83% N_2, and 7% O_2, and proliferation was assessed by the uptake of [^{3}H]thymidine (TdR) (New England Nuclear, Boston, MA) added during the last 18–24 h of a 96-h incubation period. Data are expressed as mean counts per minute (cpm) of four replicate cultures with the standard error of the mean (SEM). Net cpm (E-C) were calculated by subtracting cpm of cultures containing stimulator cells syngeneic to the responder strain (C) from cpm of cultures containing allogeneic stimulator cells (E).

Percent MLR suppression was calculated as follows:

$$\% \text{ MLR suppression} = 1 - \frac{\begin{array}{c}\text{(E-C) of MLR containing MLR-TsF or}\\ \text{regulator cells precultured with MLR-TsF}\end{array}}{\begin{array}{c}\text{(E-C) of MLR containing MEM-2\% or}\\ \text{regulator cells precultured with MEM-2\%}\end{array}} \times 100.$$

Bulk Mixed Lymphocyte Cultures. Bulk cultures were established to assess the effect of MLR-TsF on expression and function of IL-2 receptors on alloantigen-activated cells. Viable BALB/c spleen cells and irradiated B6 spleen cells were resuspended in supplemented MEM to a concentration of 6×10^6 cells/ml each and cultured with 50% volume of MLR-TsF or MEM-2% in 75-cm^2 tissue culture flasks (Co-Star, Cambridge, MA) in 5% CO_2, 37°C environment. After a 72- or 96-h incubation period, cells were harvested, layered over Lympholyte-M (Cedarlane Laboratories Limited, Hornby, Ontario, CA) density gradient to recover viable responder cells, and tested for the ability to absorb IL-2 activity from IL-2–containing supernatants. Yield and viability of cells cultured in the presence or absence of MLR-TsF did not differ appreciably. In some experiments, responder cells harvested from cultures established in control medium were subsequently assayed for their proliferative response to IL-2 in the presence of added MLR-TsF.

Absorption of IL-2–containing Supernatants. BALB/c splenocytes were activated for 72 h with B6-irradiated stimulator cells in the presence of either control medium or MLR-TsF. After selection for viable cells on a Lympholyte gradient, 50×10^6 responder cells from each group were incubated for 2 h at 37°C with 1 ml of a 1/25 dilution of an IL-2–containing EL4 supernatant. Supernatants were then harvested and assayed for residual IL-2 activity as described below. For some experiments, MLR-TsF was incubated with 4 $\times 10^6$ HT2 cells/ml for 4 h at 37°C to remove contaminating IL-2.

Assay for IL-2 Activity. IL-2–containing supernatants were assayed directly for ability to stimulate proliferation of the IL-2–dependent cell line HT2, originally described by Dr. J. Watson, University of California, Irvine, CA (18) and obtained from Drs. Kappler and Marrack, National Jewish Hospital and Research Center, Denver, CO. HT2 cells were washed thoroughly, resuspended in supplemented RPMI-1640, and 5×10^5 cells were cultured in the presence of serial, twofold dilutions of the IL-2–containing supernatant as previously described (19). TdR incorporation was determined during the last 4 h of a 24-h culture period. Activity is presented here as cpm vs. dilution of IL-2–containing supernatant.

Results

Effects of Exogenous IL-2 on MLR-TsF–induced Suppression. To determine whether MLR-TsF–mediated suppression of the proliferative response was due

to MLR-TsF associated limitation of available IL-2, we examined the possibility that the addition of excess IL-2 to those cultures would abrogate the suppressive effect. MLR consisting of BALB/c responders and irradiated B6 stimulator cells were established, various concentrations of both MLR-TsF and the IL-2–containing EL4 supernatant were added, and proliferation was measured 96 h later. The data in Fig. 1 illustrate that at each concentration of EL4 supernatant used, the suppressive effect of MLR-TsF was clearly retained. Although at higher levels of EL4 supernatant the overall proliferative response in the absence of MLR-TsF was diminished, significant MLR suppression was still observed with each concentration of MLR-TsF added. These results indicate that the suppressive capacity of MLR-TsF is not confined to a decrease in production or availability of active IL-2, and pointed to the possibility of MLR-TsF interference with IL-2 binding or utilization.

Expression of IL-2 Receptors on MLC Cells Activated in Presence of MLR-TsF. We next addressed the possibility that an altered IL-2 receptor display and/or binding capacity was occurring in MLR-TsF–suppressed cultures. Thus, responder cells from 72-h bulk BALB/c anti-B6 MLC cultures containing either 50% MLR-TsF or control medium were used to absorb IL-2 from an EL4 supernatant. The remaining IL-2 activity was assayed on the IL-2–dependent T cell line HT2 (Fig. 2). Responder cells activated in control MEM-2% and those activated in the presence of MLR-TsF demonstrated equivalent absorption capacities, reducing the dilution of EL4 supernatant required to obtain ½ maximum proliferation

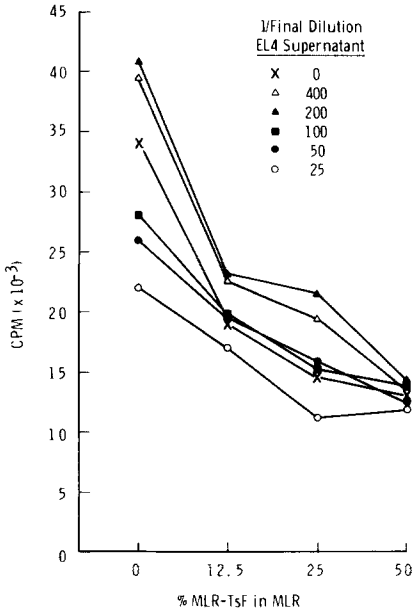

FIGURE 1. Exogenous IL-2 does not abrogate MLR-TsF–induced suppression. Mixed lymphocyte responses containing 1×10^6 BALB/c responder cells and 1×10^6 B6 stimulator cells were established in the presence of 0, 12.5, 25, and 50% MLR-TsF as indicated on the abscissa. EL4 supernatant was added to a final dilution of 0 (×), 1/400 (△), 1/200 (▲), 1/100 (■), 1/50 (●), and 1/25 (○), and proliferation was determined at 96 h.

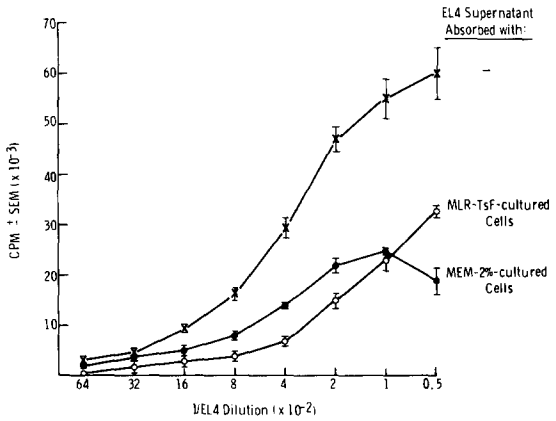

FIGURE 2. MLC cells activated in the presence of MLR-TsF express receptors for IL-2. Bulk mixed lymphocyte responses containing BALB/c responder cells and B6 stimulators were established as described in Materials and Methods. After 72 h 50 × 10^6 gradient-purified responder cells from cultures containing 50% control medium (●) or MLR-TsF (○) were incubated with a 1/25 dilution of EL4 supernatant for 2 h at 37°C. Cells were removed, and residual IL-2 activity in the supernatants as compared to unadsorbed control EL4 supernatant (×) was determined using IL-2–dependent HT2 cells as the indicator population.

from $^1/_{438}$ in the unadsorbed control to $^1/_{67}$ and $^1/_{63}$, respectively. Therefore, although the proliferative response to alloantigen was suppressed by >60% in MLR containing MLR-TsF, these results effectively demonstrate that exposure of cells to MLR-TsF during the process of alloantigen activation does not quantitatively alter the expression or binding capacity of IL-2 receptors.

MLR-TsF Activity on IL-2–dependent T Cell Proliferation. Since alloantigen activation and resultant expression of functional IL-2 receptors are not diminished by MLR-TsF, interference with binding and utilization of IL-2 was next explored as a mechanism of suppressive activity. We therefore investigated the effect of MLR-TsF on the IL-2–driven proliferative response of alloantigen–activated responder T cells and the IL-2–dependent, BALB/c-derived HT2 cell line, both already expressing surface IL-2 receptors. In panel *A* of Fig. 3, BALB/c responder cells harvested from 6-d bulk MLC were plated in microcultures with serial dilutions of EL4 supernatant and several concentrations of MLR-TsF. Proliferation measured 24–48 h later revealed significant dose-related suppression in all cultures receiving MLR-TsF regardless of the IL-2 concentration. Similarly, in panel *B* of Fig. 3, the suppressive capacity of MLR-TsF was assayed on the proliferative response of HT2 cells by titrating both MLR-TsF and EL4 supernatant into cultures containing 5 × 10^3 HT2 cells. The overall IL-2–driven HT2 proliferative response was substantially inhibited, with maximum levels of responsiveness decreasing as the level of MLR-TsF was increased. If MLR-TsF was directly affecting the IL-2–receptor interaction, a series of right-shifted curves, each attaining control-level responsiveness at high IL-2 concentrations, would be predicted. However, it is evident that for both HT2 and MLC responder cells, control-level plateau responses were never reached in the presence of MLR-TsF regardless of the availability of substantial amounts of IL-2 and that the dilution of EL4 supernatant required to obtain ½ maximum cpm

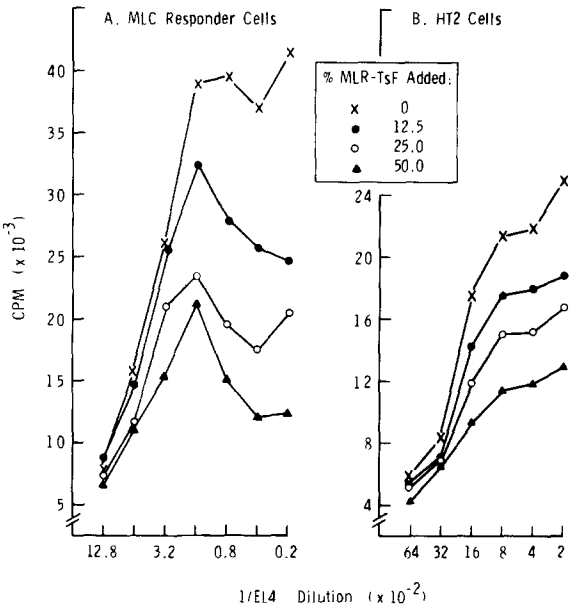

FIGURE 3. MLR-TsF suppression of IL-2 receptor-bearing responder cells. *A*, 2.5×10^5 BALB/c responder cells from a 6-d MLC or *B*, 5×10^3 HT2 cells were plated in 96-well microtiter plates in the presence of serial doubling dilutions of the EL4 supernatant (as indicated on the abscissa) and 0 (×), 12.5 (●), 25 (○), or 50% (▲) MLR-TsF. [^{3}H]Thymidine incorporation was determined 24 h later. In *A*, maximum cpm were 41,452, 32,639, 23,442, 21,274 and EL4 dilutions giving 50% maximum responses were 1/489, 1/701, 1/776, and 1/778 for 0, 12.5, 25, and 50% MLR-TsF, respectively. Similarly, in *B*, maximum cpm were 24,809, 18,765, 16,748, 13,000, and 50% maximum EL4 dilutions were 1/2542, 1/3127, 1/3082, and 1/3581 for 0, 12.5, 25.0 and 50% MLR-TsF, respectively.

for each curve remained similar (see figure legend, Fig. 3). Taken together, the results shown in panels *A* and *B* demonstrate that MLR-TsF exerts a potent dose-related suppression of cells already expressing receptors for IL-2, and suggest that MLR-TsF effects are distinct from IL-2–receptor intervention.

MLR-TsF Activity on IL-2–pulsed HT2 Cells. The possibility that MLR-TsF was interfering with the IL-2-receptor interaction either by direct competition or by a mechanism of steric hindrance was more directly addressed in the experiment shown in Fig. 4. HT2 cells were preincubated with saturating levels of IL-2 under conditions previously shown to result in maximum binding of radiolabeled IL-2 (20). These cells were then assayed for response to IL-2 in the presence of 50% MLR-TsF. Responder cells preincubated with IL-2 were subject to MLR-TsF–mediated suppression equal in magnitude to that of unpulsed HT2 cells, thus indicating an MLR-TsF-cell interaction occurring independently of the IL-2-receptor binding site. Although MLR-TsF binding to an IL-2 receptor–associated structure (21) is not ruled out, these data, in concert with those of the previous experiments, strongly suggest that the suppressive event occurs subsequent to the IL-2–receptor interaction.

Effects of Exogenous IL-2 on Ts2–Mediated Suppression. MLR-TsF suppression of HT2 cells presented in Figs. 3 and 4 provide evidence for a direct interaction with IL-2–driven responder cells that apparently bypasses the role of second-

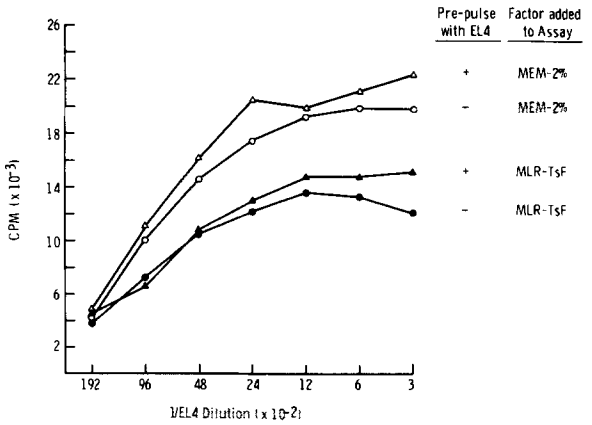

FIGURE 4. Prepulsing HT2 cells with IL-2 does not decrease MLR-TsF–induced suppression. HT2 cells were washed three times with HBSS, incubated for 1 h at 4°C with (△, ▲) or without (○, ●) saturating dilution of IL-2–containing EL4 supernatant. These cells were then plated at 5×10^3/well in the presence of serial doubling dilutions of EL4 supernatant and control medium (△, ○) or MLR-TsF (▲, ●). Proliferation was determined 24 h later.

order suppressor cells in overall suppressive activity. To further define these separable suppressive activities with regard to regulation by IL-2, we investigated the possibility that Ts2-mediated suppression might result from limited production or competition for free IL-2 in MLR, a mechanism observed in other suppressor cell systems (22–24). Thus, Ts2 or control cells were added as regulator cells to BALB/c anti-B6 MLR with several concentrations of an IL-2–containing supernatant. The results shown in Fig. 5 demonstrate a moderate, dose-related decrease in Ts2 suppressive activity with increasing concentrations of the IL-2–containing supernatant. This suggested that a component of Ts2-mediated suppression might result from limitation of available IL-2 and could be overcome under conditions of excess IL-2. However, in this experiment as well as in those done using EL4 supernatant as an IL-2 source, significant suppression of the proliferative response was always observed in Ts2–containing cultures, even in the presence of high levels of exogenous IL-2. Therefore, a major portion of Ts2-mediated suppression cannot be explained by reduced endogenous IL-2, although this mechanism does appear to contribute an element to the overall Ts2 suppressive effect. In addition, these results do not rule out a limitation of IL-2 production occurring in conjunction with other inhibitory effects.

Role of IL-2 in Ts2 Generation. We next examined the possibility that suppression attributed to limited IL-2 in the previous figure might reflect consumption of IL-2 by second-order suppressor cells during their MLR-TsF–induced activation and expansion. MLR-TsF supernatant used in Ts2 induction contain, in addition to the suppressive activities, demonstrable IL-2 (M. Beckwith, unpublished observation). This is removed by absorption with HT2 cells under conditions that do not interfere with the overall MLR suppressive capacity of the supernatants. In the experiments shown in Fig. 6, Group A, Ts2 induction cultures were established by incubating BALB/c spleen cells with MEM-2%,

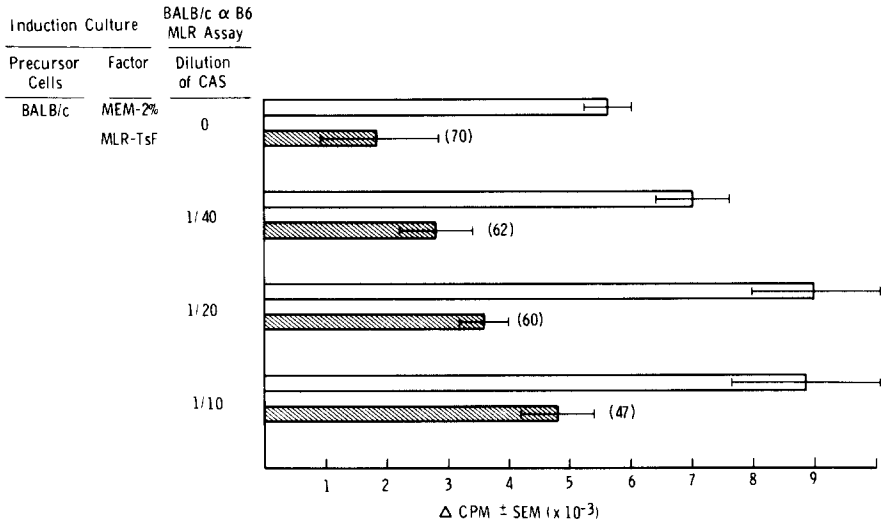

FIGURE 5. Exogenous IL-2 does not abrogate Ts2-mediated suppression. Unprimed BALB/c splenocytes were cultured for 48 h in the presence of 50% control medium (□) or MLR-TsF (▨). These cells were washed, irradiated, and added as regulator cells to BALB/c anti-B6 mixed lymphocyte responses along with the indicated dilution of Con A supernatant as a source of IL-2. Data are expressed as E-C values as described in Materials and Methods, and numbers in parentheses represent percent inhibition of suppressed vs. control cultures.

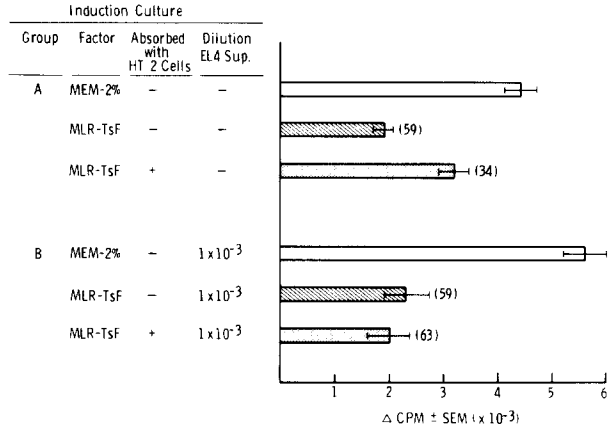

FIGURE 6. Adsorption of MLR-TsF with HT2 cells decreases Ts2 induction. Group A—Unprimed BALB/c splenocytes were cultured with 50% control medium (□), MLR-TsF (▨), or MLR-TsF that had been preadsorbed with HT2 cells to remove any contaminating IL-2 (▩). In addition, induction cultures in Group B contained a 1/1,000 dilution of EL4 supernatant as a source of IL-2. After a 48-h culture period, cells were irradiated and added to BALB/c anti-B6 MLR. Proliferation, measured at 96 h, is expressed as E-C values, and numbers in parentheses represent percent inhibition of suppressed vs. control cultures.

unadsorbed MLR-TsF, or MLR-TsF that had been depleted of IL-2 by preadsorption with HT2 cells. The cultures in Group B contained, in addition, a 1/1,000 dilution of the IL-2–containing EL4 supernatant. In Groups A and B, MLR receiving regulator cells that had been cultured with unabsorbed MLR-TsF were each suppressed by 59%. This suppression was reduced by >40% in

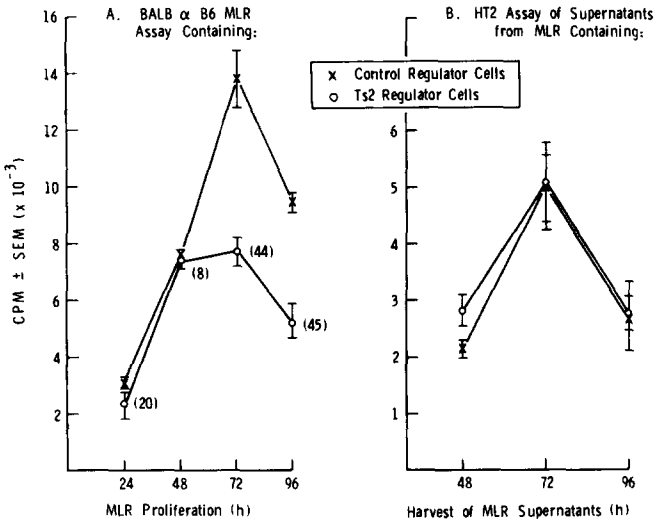

FIGURE 7. Kinetics of Ts2 suppression and IL-2 production in Ts2-regulated MLR. MLR were established containing 5×10^5 BALB/c responder cells, 5×10^5 B6 stimulator cells and 5×10^5 regulator cells that had been generated in the presence of 50% control medium (×) or MLR-TsF (○). In *A*, proliferation of the regulated MLR was measured at 24, 48, 72, and 96 h. The numbers in parentheses represent percent inhibition of Ts2 vs. control cultures. In *B*, supernatants from identical cultures that had not been labeled with [^{3}H]thymidine were taken at each time point, and were tested for IL-2 activity by 24-h assay with IL-2–dependent HT2 cells.

MLR receiving regulators that had been generated with IL-2–depleted MLR-TsF (Group A), and was completely restored when a low level of IL-2 was included in the induction cultures (Group B, closed bar). These results indicate that maximal Ts2 induction involves a molecule that both binds to HT2 cells and is present in an EL4 supernatant. Since Ts2 induction was never completely blocked when using IL-2–depleted MLR-TsF, these data are consistent with a synergistic role for more than one lymphokine, including IL-2, in Ts2 induction. Alternatively, a specific suppressor cell inducing moiety in the EL4 supernatant may contribute to suppressor cell growth. Taken together, the results shown in Figs. 5 and 6 demonstrate that while second-order suppressor cells primarily inhibit alloantigen-induced proliferation by a mechanism other than consumption of endogenously produced IL-2, they may require IL-2 during the inductive process, and therefore competitively remove a fraction of IL-2 from responder cell use.

IL-2 Production in Ts2-Suppressed MLR. We next wished to directly assess the level of IL-2 produced in cultures undergoing Ts2-mediated suppression. Ts2 or control regulator cells were added to fresh BALB/c anti-B6 MLR, and at 24, 48, 72, and 96 h after culture initiation, suppression was monitored by [^{3}H]thymidine uptake (Fig. 7*A*), and supernatant samples were taken for determination of IL-2 content in the HT2 assay (Fig. 7*B*). In Fig. 7*A*, the 72-hour peak response in both the control and Ts2-regulated MLR demonstrate that Ts2-mediated suppression is not simply due to a shift in the kinetics of proliferation. The kinetics of suppression also indicate a late-acting effect, with minimum

suppression observed at 48 h, increasing to maximum levels by 96 h after culture initiation. This suggests, as was observed with MLR-TsF (4), that Ts2-mediated suppression does not appear to interfere with early antigen recognition and activation events, but could involve a limitation in subsequent production of IL-2 or utilization of IL-2 during the course of the response, thus limiting the proliferative response. However, in Fig. 7*B*, it is quite clear that equivalent levels of IL-2 were measured at each time point in both control and Ts2-regulated MLR, thus directly demonstrating that IL-2 production is not limited in Ts2 containing MLR. These data support the concept that Ts2-mediated suppression, as observed for MLR-TsF, may occur by inhibition of proliferative signals manifest at a later point in the interleukin cascade.

Discussion

In this report we investigated the possibility that MLR-TsF suppresses alloantigen-stimulated T cell responses by interference with IL-2–mediated regulation of T cell proliferation. The present data demonstrate that one element of MLR-TsF–mediated suppression involves inhibition of IL-2–driven proliferation of activated responder T cells. This inhibition does not result from either limited availability of IL-2 in the suppressed culture, or decreased expression of functional IL-2 receptors, but appears primarily to involve interference with a proliferative event occurring subsequent to the IL-2–receptor interaction. Furthermore, this direct inhibitory effect is clearly distinguishable from the Ts2 inductive capacity of MLR-TsF. In this report we demonstrate that Ts2-mediated suppression is comprised of at least two components which together produce a late-acting inhibitory effect peaking 72-h after culture initiation. A minor component was overcome by exogenous IL-2, and may reflect a requirement for IL-2 during Ts2 expansion. However, the most significant element of suppression could not be explained by direct competition for IL-2, or by limited production of IL-2 in the MLR, and thus results from a defect in later phases of the proliferative response.

Since excess levels of exogenous IL-2 failed to abrogate MLR-TsF–mediated suppression, inhibition could not be explained solely by lack of available IL-2. This does not rule out the possibility that an MLR-TsF–induced defect in IL-2 production occurs in conjunction with other inhibitory activities. Kramer and Koszinowski (25) describe a 10,000-dalton column purified protein from an alloantigen-induced T cell supernatant that suppresses T cell responses entirely by inhibiting the production of IL-2. However, in our system, attempts to directly measure IL-2 production in suppressed cultures was complicated by the inhibitory effect of MLR-TsF in the HT2 assay used for IL-2 quantitation. Thus, the additional possibility that a decrease in IL-2 production contributes to overall MLR-TsF suppression remains untested.

Several observations in this report support the concept of MLR-TsF interference with proliferative events occurring subsequent to IL-2 receptor binding. First, IL-2 absorption experiments demonstrated that alloantigen-induced expression and binding capacity of IL-2 receptors were not limited by MLR-TsF. Second, alloantigen-activated T cells and HT2 cells, both expressing functional IL-2 receptors, were susceptible to MLR-TsF suppression, and third, this

suppression was still observed after presaturation of IL-2 receptors with excess IL-2. If these results are examined in the light of models of hormone receptor binding proposed for certain types of insulin resistance (26, 27), an interesting pattern emerges. Proposed mechanisms of resistance to insulin can be divided into prereceptor, receptor, or postreceptor defects. In each case characteristic dose-response curves demonstrating either a decreased sensitivity to insulin, a decreased responsiveness, or a combination effect can be utilized to predict which mechanism is being observed. Thus, decreased sensitivity to insulin is characterized by right-shifted curves, each of which ultimately reaches the maximum control level of proliferation at increased hormone concentrations, and would be observed in the presence of abnormal insulin molecules or anti-insulin antibodies. On the other hand, patterns of decreased responsiveness to insulin are manifest by dose-response curves that never reach the maximum biological response seen in controls, and that require the same concentration of hormone to obtain 50% of the maximum response for each curve. For insulin this pattern is typically associated with defects in which the abnormal response is localized to intracellular events that follow the hormone receptor interaction, and is thus classified as a postreceptor defect (26–27). The profile of IL-2–stimulated proliferation observed in the presence of MLR-TsF (Figs. 3 and 4) clearly resembles the latter pattern of decreased responsiveness to IL-2. Thus maximal proliferation was never obtained in suppressed cultures, and the concentration of IL-2 required to yield one-half maximum response was quite similar for each curve. This pattern might also be observed if an overall decrease in IL-2 receptors was induced by MLR-TsF, however, this possibility is ruled out by absorption experiments that indicate no MLR-TsF effect on IL-2-receptor expression. Therefore, the observed response is most consistent with a defect occurring after the IL-2 receptor interaction, i.e. a postreceptor defect.

In the increasingly complex cascade of molecular events ultimately resulting in T cell proliferation, initial T cell activation by mitogen or antigen promotes IL-1 release by monocytes followed by T cell production of IL-2 and expression of IL-2 receptors. In contrast, those steps occurring after the IL-2–receptor interaction are less clear, and stimulation of additional cell-surface molecules may be required before DNA synthesis. Receptors for the iron-binding glycoprotein transferrin appear on activated human T cells only after IL-2 receptor expression and binding, and the transferrin-receptor interaction is required for cell proliferation (28). In addition, Malek et al. (21) have described a monoclonal antibody that recognizes a guinea pig T cell surface antigen that is distinct from the IL-2 receptor but required for IL-2–driven proliferation, thus possibly providing a second growth signal following IL-2 binding. MLR-TsF suppression may involve interference with a cell surface molecule similarly associated with IL-2–receptor binding, but critical in the lymphokine cascade only after IL-2 stimulation has occurred.

Alternatively, the target of inhibition could be intracellular events involved in IL-2-receptor internalization, processing in lysosomal vesicles (20) or generation of Ca^{++}- or cAMP-mediated second signals required for proliferation. Palacios and Martinez-Maza (29) reported that interference with Ca^{++} uptake by OKT11 monoclonal antibody completely turns off processes of T cell activation including

production of IL-2 and IL-2 receptor expression. It is therefore unlikely that MLR-TsF acts as a negative signal through interference with early Ca^{++}-dependent events. Binding assays utilizing radiolabeled IL-2 and MLR-TsF preparations in conjunction with anti-receptor antibodies will more precisely define the site of MLR-TsF activity.

Direct MLR-TsF inhibition of HT2 cell proliferation to IL-2 is consistent with a model of two independent mechanisms of MLR-TsF–mediated suppression, comprised of direct MLR-TsF interference with proliferating target cells subsequent to IL-2 binding, and initiation of the Ts2 pathway. Alternatively, MLR-TsF suppression of HT2 cell responsiveness could be due to a soluble factor within the MLR-TsF preparation that is produced by early Ts2 during the MLR-Ts in vitro restimulation period, thus supporting the concept of a sequential rather than simultaneous model for MLR-TsF–induced suppression. Production of a nonspecific inhibitor of DTH has been described that follows the genetically restricted interaction of an $I\text{-}J^+$ suppressor factor with a T acceptor cell, and that functions through inhibition of IL-2 production (8, 9). Kinetics of MLR-Ts2 suppression would be consistent with release of a molecule inhibiting later events in the proliferative phase of clonal expansion rather than initial activation or differentiation to an IL-2 responsive state. However, HT2 assay of supernatants from control and Ts2-regulated cultures indicates that if Ts2 cells suppress via a late-acting soluble factor, it does not function by decreasing IL-2 production or, more importantly, by inhibiting IL-2–driven proliferation of HT2 cells. This lack of suppressive effect on HT2 cells, as opposed to the inhibition observed with MLR-TsF, argues against a single sequential pathway of MLR-TsF suppression and instead supports the existence of distinct suppressive molecules, one acting directly on proliferating cells, and another resulting in Ts2 induction and subsequent inhibition.

The precise mechanism of MLR-Ts2–mediated suppression remains unclear, however it appears that more than one process contributes to the overall effect. Sy and co-workers (2) described two mechanisms of suppression leading to decreased hapten-specific CTL responses that could be distinguished by their susceptibility to excess IL-2. Competitive consumption of IL-2 by added regulator cells has been clearly demonstrated for both alloantigen (22, 23) and Con A–activated suppressor T cells (24). Similarly, modest reconstitution of Ts2-suppressed MLR by exogenous IL-2 suggested a degree of Ts2-associated consumption of IL-2. This appeared to derive from the utilization of IL-2 during Ts2 induction and expansion rather than from direct limitation of IL-2 production. However, it is clear that the most significant component of Ts2-mediated suppression is not susceptible to exogenous IL-2, and may therefore involve interference with later events dependent on IL-2 utilization.

In summary, the predominant mechanism of alloantigen-induced T cell suppression in the present system occurs by interference with IL-2 utilization in the face of largely unimpaired IL-2 receptor display or IL-2 production. Direct interference with IL-2–driven proliferation by MLR-TsF has been isolated more precisely to events that occur only after IL-2–receptor binding, and is strikingly consistent with a prediction of induced postreceptor defect of hormone-receptor interaction. However, the relative roles of inhibited IL-2–dependent activity

mediated directly by MLR-TsF and indirectly via Ts2 induction, and their individual control by restricting MHC elements remains to be determined, and will contribute an important dimension to the overall profile of alloantigen-induced suppression.

Summary

In this report we examined the possibility that suppression of the mixed lymphocyte response by MLR-TsF results from interference with IL-2 regulation of T cell proliferation. Two distinct processes of inhibition involving both a direct effect on IL-2–driven proliferation of responder T cells, and induction of a second-order suppressor cell (Ts2) were described. Exogenous IL-2 did not abrogate MLR-TsF–induced suppression, and activated responder cells from suppressed cultures expressed functional IL-2 receptors by IL-2 adsorption analysis. Thus, suppression is not due to lack of available IL-2 or to abnormal acquisition of receptors for IL-2 during T cell activation. In contrast, a profound MLR-TsF effect on IL-2–induced proliferation of HT2 cells as well as MLR-activated cells was observed even after presaturation of receptors with excess IL-2. These results differentiated the direct responder cell effect of MLR-TsF from its Ts2 inductive capacity, and localized the defect in responder cell proliferation to events occurring subsequent to IL-2 binding. When analyzed in terms of proposed models for hormone–receptor interactions, characteristic dose-response curves similarly predict a postreceptor defect.

Examination of the Ts2 pathway of suppression revealed a late-acting inhibitory effect peaking 72 h after MLR initiation. A minor part of Ts2 activity was susceptible to exogenous IL-2, and may reflect a requirement for IL-2 during Ts2 expansion. However, the most significant component of Ts2-mediated suppression was resistant to excess IL-2, and IL-2 production was normal in Ts2-regulated cultures, thus ruling out limitation of IL-2 for responder cell use as the major mechanism of Ts2 suppression. The complete pathway of Ts2 suppression and its functional relationship to other MLR-TsF inhibitory activities is not yet fully understood. However, these results suggest that the ultimate mechanisms of alloantigen-induced suppression involve late events of the IL-2–dependent lymphokine cascade.

We thank Mrs. Sue Floyd for excellent secretarial assistance.

Received for publication 11 August 1983.

References

1. Hardt, C., M. Röllinghoff, K. Pfizenmaier, H. Mosmann, and H. Wagner. 1981. Lyt-2^+3^+ CY-sensitive T cells regulate the activity of an interleukin 2 inhibitor in vivo. *J. Exp. Med.* 154:262.
2. Sy, M. S., S.-H. Lee, M. Tsurufuji, K. L. Rock, B. Benacerraf, and R. Finberg. 1982. Two distinct mechanisms regulate the in vivo generation of cytotoxic T cells. *J. Exp. Med.* 156:918.
3. Gautum, S. C., M. L. Hilfiker, and J. R. Battisto. 1983. In vivo development of cytolytic T lymphocytes (CTL) to hapten-altered self: MLS-disparate cells facilitate the response by neutralizing IL-2 inhibitor. *J. Immunol.* 130:533.

4. Rich, S. S., and R. R. Rich. 1975. Regulatory mechanisms in cell-mediated immune responses. II. A genetically restricted suppressor of mixed lymphocyte reactions released by alloantigen-activated spleen cells. *J. Exp. Med.* 142:1391.
5. Rich, S. S., and R. R. Rich. 1976. Regulatory mechanisms in cell-mediated immune responses. III. I-region control of suppressor cell interaction with responder cells in mixed lymphocyte reactions. *J. Exp. Med.* 143:672.
6. Rich, S. S., C. S. David, and R. R. Rich. 1979. Regulatory mechanisms in cell-mediated immune responses. VII. Presence of I-C subregion determinants on mixed leukocyte reaction suppressor factor. *J. Exp. Med.* 149: 114.
7. Beckwith, M., and S. S. Rich. 1982. Suppressor-target interaction in alloantigen induced responses: induction of a second cell in the suppressive pathway. *J. Immunol.* 128:791.
8. Zembala, M. A., G. L. Asherson, B. M. B. James, V. E. Stein, and M. C. Watkins. 1982. Anti-haptene T suppressor factor acts through an I-J$^+$, Ly-1$^-$2$^+$, T acceptor cell that releases a nonspecific inhibitor of the transfer of contact sensitivity when exposed to antigen. *J. Immunol.* 129:1823.
9. Malkovsky, M., G. L. Asherson, B. Stockinger, and M. C. Watkins. 1982. Nonspecific inhibitor released by T acceptor cells reduces the production of interleukin-2. *Nature (Lond.).* 300:652.
10. Eardley, D. D., J. Hugenberger, L. McVay-Boudreau, F. W. Shen, R. K. Gershon, and H. Cantor. 1978. Immunoregulatory circuits among T-cell sets. I. T-helper cells induce other T-cell sets to exert feedback inhibition. *J. Exp. Med.* 147:1106.
11. Sy, M. S., M. H. Dietz, R. N. Germain, B. Benacerraf, and M. I. Greene. 1980. Antigen and receptor-driven regulatory mechanisms. IV. Idiotype-bearing I-J$^+$ suppressor T-cell factors induce second-order suppressor T-cells which express anti-idiotypic receptors. *J. Exp. Med.* 151: 1183.
12. Germain, R. N., and B. Benacerraf. 1981. Hypothesis. A single major pathway of T-lymphocyte interactions in antigen-specific immune suppression. *Scand. J. Immunol.* 13:1.
13. Wagner, H., and M. Röllinghoff. 1978. T-T interactions during in vitro cytotoxic allograft responses. I. Soluble products from activated Ly-1$^+$ T cells trigger autonomously antigen primed Ly-2$^+$3$^+$ T cells to cell proliferation and cytolytic activity. *J. Exp. Med.* 148:1523.
14. Smith, K. A., L. B. Lachman, J. J. Oppenheim, and M. F. Favata. 1980. The functional relationship of the interleukins. *J. Exp. Med.* 151:1551.
15. Gillis, S., G. R. Crabtree, and K. A. Smith. 1979. Glucocorticoid-induced inhibition of T cell growth factor production. II. The effect on the in vitro generation of cytolytic T cells. *J. Immunol.* 123:1632.
16. Farrar, J. J., J. Fuller-Farrar, P. L. Simon, M. L. Hilfiker, B. M. Stadler, and W. L. Farrar. 1980. Thymoma production of T cell growth factor (interleukin 2). *J. Immunol.* 125:2555.
17. Spiess, P. J., and S. A. Rosenberg. 1981. A simplified method for production of murine T-cell growth factor free of lectin. *J. Immunol. Methods.* 42:213.
18. Watson, J. 1979. Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. *J. Exp. Med.* 150:1510.
19. Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.
20. Robb, R. J., A. Munck, and K. A. Smith. 1981. T cell growth factor receptors: quantitation, specificity, and biological relevance. *J. Exp. Med.* 154:1455.
21. Malek, T. R., R. J. Robb, and E. M. Shevach. 1983. Identification of a membrane antigen that is distinct from the interleukin-2 receptor and that may be required for

interleukin-2 driven proliferation. *J. Immunol.* 130:747.
22. Susskind, B. M., V. J. Merluzzi, R. B. Faanes, M. A. Palladino, and Y. S. Choi. 1983. Regulatory mechanisms in cytotoxic T lymphocyte development. I. A suppressor T cell subset that regulates the proliferative stage of CTL development. *J. Immunol.* 130:527.
23. Gunther, J., W. Haas, H. Von Boehmer. 1982. Suppression of T cell responses through competition for T cell growth factors (interleukin 2). *Eur. J. Immunol.* 12:247.
24. Palacios, R., and G. Möller. 1981. T cell growth factor abrogates concanavalin A induced suppressor cell function. *J. Exp. Med.* 153:1360.
25. Kramer, M., and V. Koszinowski. 1982. T cell specific suppressor factors with regulatory influence on interleukin-2 production and function. *J. Immunol.* 128:784.
26. Olefsky, J. M., and O. G. Kolterman. 1981. Mechanisms of insulin resistance in obesity and non-insulin-dependent (Type II) diabetics. *Am. J. Med.* 70:151.
27. Rizza, R. A., L. J. Mandarino, and J. E. Gerich. 1981. Mechanisms of insulin resistance in man: assessment using the insulin dose-response curve in conjunction with insulin-receptor binding. *Am. J. Med.* 70:169.
28. Neckers, L. M., and J. Cossman. 1983. Transferrin receptor induction in mitogen-stimulated human T lymphocytes is required for DNA synthesis and cell division and is regulated by interleukin-2. *Proc. Natl. Acad. Sci. USA.* 80:3494.
29. Palacios, R., and O. Martinez-Maza. 1982. Is the E receptor on human T lymphocytes a "negative signal receptor"? *J. Immunol. 129:2479.*