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Immunoregulation by Mouse T-Cell Clones

I. Suppression and Amplification of Cytotoxic Responses by Cloned H-Y-Specific Cytolytic T Lymphocytes

MARKUS M. SIMON,[1] HEIDRUN MOLL, MARLOT PRESTER, GABY NERZ, AND KLAUS EICHMANN

Max-Planck-Institute for Immunobiology, Stübeweg 51, D-7800 Freiburg, Federal Republic of Germany

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H-Y-specific and H-2D^b-restricted, Lyt-1$^-$2$^+$ T-cell clones (CTLL) with graded specific cytotoxic activities on male C57BL/6 (B6) target cells (1E3, +++; 2C5, ++; 2A5, +, 3E6, ±) were tested for their capacity to inhibit the generation of H-Y-specific cytotoxic T lymphocytes (CTL) *in vitro*. Addition of irradiated lymphocytes of CTLL 1E3 and CTLL 3E6 but not those of CTLL 2A5 or CTLL 2C5 abolished the generation of CTL from *in vivo* primed H-Y-specific precursor cells (CTLP) when added to fresh mixed-lymphocyte cultures (MLC). Exogenous sources of T-cell growth factors (TCGF) did not overcome suppression. Rather the presence of TCGF resulted in a further enhancement of suppressive activities in CTLL 1E3 and 3E6 and the induction of similar activities in cells from CTLL 2A5 and 2C5, which by themselves were not inhibitory. Moreover when added to similar MLC on Day 1 instead of Day 0, only irradiated cells of CTLL 3E6 but not those of the other three CTLL were suppressive. Induction of suppressive activities in H-Y-specific CTLL was independent of the appropriate male stimulator cells since it was also observed in MLC induced by irrelevant antigens (H-2, trinitrophenol). Furthermore at low cell numbers, irradiated lymphocytes from any of the CTLL consistently enhanced CTL activities generated from H-Y-specific CTLP. This augmenting activity, which was not TCGF, could be transferred by soluble mediators present in antigen-sensitized CTLL cultures. Thus, these data indicate (*i*) that cytotoxic effector cells can function as suppressor cells in the generation of CTL, (ii) that the cytotoxic activity of cloned CTL does not correlate with their capacity to suppress CTL responses, (iii) that the inhibition of CTL responses by CTLL is not due to simple consumption of T-cell growth factors produced in MLC, and (iv) that different CTL clones may interfere with the generation of CTL at different stages of their maturation. Moreover, the experiments suggest an antigen-independent enhancement of suppression by the interaction of CTL with lymphokines. Together with the augmenting activity evoked by cloned CTL the data provide strong evidence for the expression of multiple immunological functions by one particular subset of T cells and suggest that cytotoxic effector cells can differentially regulate the maturation and/or clonal expression of their precursor cells.

INTRODUCTION

It is generally accepted that the generation and regulation of effector functions in the immune system require interactions between functionally distinct lymphocyte

[1] To whom correspondence should be addressed.

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subsets. Thus, during the development of cytotoxic T lymphocytes (CTL)[2] in mixed-lymphocyte cultures one observes the concomitant appearance of various T-cell functions involved in the regulation of CTL responses such as help (TH) (1–3) and suppression (TS) (4–19) which can be tested upon transfer to fresh mixed-lymphocyte cultures (MLC). Helper and cytotoxic activities have been shown to be functions of two separate T-cell subsets which are distinguishable by their Lyt phenotypes (20, 21) as well as by their restricted recognition of different MHC-encoded structures (22, 23). In contrast, a clear definition of T cells involved in suppression of cytotoxic responses has not been achieved. This is because TS and CTL share several characteristics like the expression of Lyt 23 alloantigens (13, 15, 21) and the preferential reaction with H-2K or H-2D antigens (24, 25). Two suppressor functions can be distinguished in MLC populations, one which specifically (4, 5, 8, 10, 12, 13, 15, 17) and one which nonspecifically (7, 8, 10, 11, 14, 16, 18, 19) suppresses the generation of new CTL in fresh MLC. The nonspecific TS could be differentiated in some reports (8, 10, 11, 14, 16) from the antigen-specific TS and from CTL by its susceptibility to irradiation or treatment with mitomycin. However, in other studies (18, 19; M. M. Simon unpublished results) nonspecific suppression of the generation of CTL has also been demonstrated with irradiated MLC-activated lymphocyte populations so that CTL and both suppressive activities remained in the radioresistant MLC-derived population.

In a multitude of studies MLC-activated T suppressor cells have been implied to be either distinct or identical to the CTL on the basis of irradiation sensitivity and elicitation of effector mechanisms (4–8, 10–19), serological analyses (13, 15, 21), antigenic requirements (12, 13), inducibility by drugs (7, 13, 17), and kinetics of their development (7). Some but not all of these conflicting results may be explained by differences in the protocols used for the induction and determination of suppressive activity as well as for the selection procedures by antisera and complement.

On the basis of these investigations a variety of mechanisms for suppression of CTL development have been proposed including elimination or inactivation of stimulator cells (4, 5, 15), inactivation of precursor cells of CTL (CTLP) (6–8, 13), or the inhibition of clonal expansion of activated CTLP by depletion of lymphokines, such as T-cell growth factors, from the cultures (18, 19, 26). However, none of these three possibilities alone has been formally proven to be the only reason for the suppressive activity of MLC-activated lymphocyte populations. In addition, the question of whether antigen-specific and nonspecific suppressor T cells belong to defined T-cell subsets remains unanswered.

In none of the aforementioned studies cytotoxic and suppressor T cells have been clearly separated and, therefore, the possibility remained that both functions were exerted by one type of T cell. In this report we therefore examine the suppressive activity of cloned antigen-specific cytotoxic T cells established in long-term tissue culture. We describe the results of a detailed characterization of the regulatory functions of T-cell clones expressing various degrees of cytolytic activities and maintained in

[2] Abbreviations used: MLC, mixed-lymphocyte culture(s); CTLL, cloned cytotoxic T-cell line; CTL, cytotoxic T lymphocyte(s); CTLP, precursor cell(s) of cytotoxic T lymphocytes; TH, T helper cell(s) for generation of CTL; TS, T suppressor cell(s) of cytotoxic responses; B6, C57BL/6 mice; Con A, concanavalin A; PHA, phytohemagglutinin; Con A-SN, supernatant of Con A-induced rat spleen cells; MLC-SN, supernatant of secondary mouse MLC, TCGF, T-cell growth factor; TNBS, trinitrobenzenesulfonate; α-MM, α-methy-D-mannoside; TdR, thymidine.

continuous culture for periods of up to 12 months. The data demonstrate that cloned cytotoxic T lymphocytes and their long-term culture derivatives have a strong suppressive effect on the induction of cytotoxic T lymphocytes *in vitro*. Different T-cell clones may use different mechanisms for suppression. In addition, the results suggest that low numbers of the same cloned cytotoxic T lymphocytes are able to enhance the induction of cytotoxic responses.

MATERIALS AND METHODS

Mice. Adult mice of the inbred strains C57BL/6 (B6), BALB/c, and DBA/2 were obtained from the animal colony maintained at the Max-Planck-Institute for Immunobiology, Freiburg, Federal Republic of Germany.

Preparation of culture supernatants. Preparation of Con A supernatants (Con A-SN) from rat spleen cells has been described in detail previously (27). Briefly, Con A-SN was prepared by stimulating spleen cells from Spraque–Dawley rats with Con A (5 μg/ml) at 5×10^6/ml in complete RPMI 1640 medium. Supernatant fluids were collected after 24 hr of incubation and supplemented with 20 mg/ml of α-methyl-D-mannoside (α-MM; Sigma Chemical Co., Munich, FRG).

Secondary (2°) MLC supernatant (MLC-SN) was prepared as described by Ryser *et al.* (28), by mixing 1×10^7 viable cells recovered from pools of 14-day-old primary BALB/c anti-DBA/2 MLC with 4×10^6 irradiated (2000 rad) DBA/2 spleen cells in 10 ml of culture medium in small tissue culture flasks (25 cm^2/50 ml; 163371, Nunc, Wiesbaden, FRG). Supernatants were collected after 24 hr of incubation and sterilized by filtration.

The efficiency of each supernatant preparation was determined by support of the proliferation of various *in vitro* T-cell lines (clone 96; 3E6) and by their ability to induce primary cytotoxic lymphocytes. All experiments described in this paper were done with supernatant preparations giving optimal support in both detector systems at dilutions of 1:10 to 1:20.

TCGF assay. TCGF activity in Con A-SN and in MLC-SN was measured by incubating serial dilutions of supernatants (100 μl) with 1×10^3 TCGF-dependent clone 96 or 3E6 cells in a total volume of 150 μl in round-bottom microtiter plates (Greiner, Nürtingen, FRG) at 37°C. After 3 days of incubation 1.25 μCi [^{3}H]TdR was added during the last 4 hr. Cells were harvested onto filter strips using an automatic cell harvester and counted in a liquid scintillation β counter.

Cloned cytotoxic T cells. All cloned cytolytic T cells (1E3, 2A5, 2C5, 3E6) were derived from long-term MLC containing female B6 responder T cells (prepared by nylon-wool passage of spleen cells (29)) derived from mice previously primed to male B6 cells (by ip injection of 3×10^7 male B6 spleen cells) and male B6 stimulator cells as described in detail previously (30). Briefly, the H-Y-specific T-cell line was restimulated *in vitro* in 50-ml tissue culture flasks (Nunc, 163371) at weekly intervals with 2.5×10^7 irradiated male B6 stimulator cells in RPMI 1640 medium supplemented with 10% fetal calf serum, 10^{-5} *M* 2-mercaptoethanol, 2.5 m*M* Hepes, 2 m*M* glutamine, kanamycin (100 μg/ml), and tylosin (10 μg/ml) (RPMI 1640, complete) in 5% CO_2 atmosphere. From 6 weeks on, the H-Y-specific T-cell line was maintained and expanded in 50-ml tissue culture flasks on antigen in Con A-SN (10%)-conditioned medium. H-Y-specific CTL clones were obtained by limiting dilution procedures by seeding from 10 to 0.3 long-term culture responder cells/well and expanding lym-

phocytes on 5×10^5 irradiated (2200 rad) male B6 stimulator cells in Con A-SN (10–20%)-conditioned medium in round-bottom microtiter plates (Nunc, 163320). After 2–3 weeks, cytotoxic activities were tested in individual wells on ^{51}Cr-labeled Con-A-stimulated male B6 target cells and the positive lines derived from wells which received either 1 or 0.3 cell/well were picked and expanded on antigen in the presence of Con A-SN in a volume of 2 ml in macrotiter plates (Linbro 1624 TC) and subsequently in 50-ml tissue culture flasks under similar conditions. All CTL lines (CTLL) were recloned twice on antigen and Con A-SN at dilutions of 0.3 cell/well under similar conditions. Four months after the last recloning procedure, one subclone of CTLL 3E6 was adjusted to grow in Con A-SN-conditioned medium in the absence of antigen. Con A-SN-dependent CTLL 3E6 clones were obtained by limiting dilution procedures as described above. Specificities and cytotoxic activities of CTLL was tested on Con A-stimulated spleen cells of male and female B6 mice, and on P815 tumor targets in the absence or for lectin-mediated lympholysis in the presence of PHA (Gibco, 670-0576, 4% stock solution).

Assay for suppressive and augmenting activities of CTLL. Cultures containing 3×10^6 splenic responder cells from female B6 mice (previously primed to male B6 cells by ip injection of 3×10^6 B6 spleen cells) or 1×10^6 splenic responder cells from female BALB/c mice and 5×10^6 irradiated (2200 rad) spleen cells from either male or female B6 mice (unmodified or modified with TNBS, 3 m*M*, 10 min, 37°C) were incubated in the absence or presence of MLC-SN (10–20%) in a final volume of 2 ml RPMI 1640 complete medium (supplemented as described above) in macrotiter plates (Linbro 1624 TC). Various numbers (1×10^5 to 1×10^6) of irradiated (1200 rad) CTLL were added to these cultures on either Day 0 or Day 1 of the culture period. After 5 days of incubation at 37°C in a humidified atmosphere of 5% CO_2, effector cells were tested on the appropriate ^{51}Cr-labeled target cells.

Assay for lymphokine activities in culture supernatants of CTLL. Cloned cytotoxic T cells (1×10^6) were irradiated (1200 rad) and incubated with 5×10^6 irradiated (2200 rad) and T-cell-depleted (pretreatment of spleen cells with anti-Thy-1.2 plus complement) male B6 spleen cells in complete RPMI 1640 medium in a final volume of 2 ml at 37°C in a humidified atmosphere of 5% CO_2. After 24 hr, the supernatants were harvested, filtered, and stored at −20°C until use. Supernatants from cultures containing stimulator cells only served as controls. Individual supernatants were tested for their content of TCGF activity on the TCGF-dependent line 3E6 as described above. In addition the supernatants were tested for their augmenting activities of CTL responses by addition to cultures consisting of 3×10^6 spleen cells from female B6 mice (previously primed to male B6 spleen cells) and 5×10^6 irradiated (2200 rad) male B6 spleen cells. After 5 days of incubation effector activities were tested on the appropriate ^{51}Cr-labeled target cells.

Assay for cell-mediated cytotoxicity. Specific ^{51}Cr release was determined by incubating serial dilutions of effector cells derived from macrocultures with a fixed number (2×10^3/well) of ^{51}Cr-labeled Day 2 Con A-activated blasts from male or female B6 mice (unmodified or modified with TNBS), with ^{51}Cr-labeled P815 or EL-4 tumor cells in a final volume of 0.2 ml for 4 hr. Afterward plates were centrifuged and 100 μl of supernatant was removed for counting. The percentage specific lysis was calculated by using the equation: % ^{51}Cr release $= (x - y)/(z - y) \times 100$ in which x is the counts per minute in the supernatant of target cells mixed with effector

cells; y is the counts per minute in the supernatant of target cells incubated alone; z is the counts per minute after lysis of target cells in 1 N HCl.

Limiting dilution microcultures and calculation of CTLP frequencies. Microcultures of CTLL were prepared as described previously (30). Limiting numbers of cloned CTL (24 microwells/cell dose) were cultured in the presence of Con A-SN (20% end concentration) with 5×10^5 irradiated (2200 rad) male B6 spleen cells in 200 μl, in round-bottom microtiter plates (Nunc, 163320), and incubated at 37°C in a humidified atmosphere of 5% CO_2. After 7 days of incubation cultures were restimulated by removing 150 μl of supernatant and adding, in the same volume, 5×10^5 irradiated male B6 spleen cells in medium supplemented with Con A-SN (20% end concentration). After a further incubation period of 6 days cytotoxic activities were determined on Day 13 by incubating aliquots of each microwell (100–150 μl) with 2×10^3 Day 2 Con A-activated and ^{51}Cr-labeled male B6 blast cells in 200 μl for 4 hr. Plates were centrifuged and 100 μl of supernatant was removed for counting. Micro-MLC were defined as positive for CTL activity when ^{51}Cr release exceeded the mean spontaneous ^{51}Cr release by at least 3 SD. Minimal estimates of frequencies of CTLP and 95% confidence limits were determined by the minimum χ^2 method from the Poisson distribution relationship between the numbers of responder cells added per microculture and the fraction of nonresponding cultures per group (31). Those experiments with $P < 0.05$ were rejected.

RESULTS

General Properties of H-Y-Specific CTLL

All H-Y-specific T-cell clones were obtained by limiting dilution cloning from one long-term tissue culture line of female B6 responder cells originally primed *in vivo* to the H-Y antigen and repeatedly restimulated *in vitro* with male B6 spleen cells. Clones displaying high cytolytic activity at low effector cell to target cell ratios against male B6 Con A blasts were recloned twice and propagated on antigen in the presence of ConA-SN. All CTLL were Thy-1.2$^+$, Lyt-1$^-$2$^+$, and restricted for H-2D^b (data not shown). Most of these cloned lines retained high cytolytic activities for a 5-month-period in both specific and lectin-mediated test systems (see below). Limiting dilution analysis of freshly cloned T cells, an example of which is depicted in Fig. 1, revealed frequencies for CTL in CTLL of approximately (1/1.16), indicating that each individual cell within a clone gave rise to progenies expressing cytolytic activity (Fig. 1). It was also observed that the CTLL could be expanded for periods of up to 1 week on irrelevant stimulator cells in the presence of ConA-SN without losing their functional activities. During the course of multiple restimulations and irrespective of repeated recloning steps, all cloned CTLL gradually lost their specific cytolytic activity on male B6 target cells (Fig. 2), which was also reflected in their decreasing frequencies of H-Y-specific CTL, but retained their ability to mediate lectin-facilitated lympholysis (30; data not shown).

Four clones, the characteristics of which are outlined in Table 1, were finally chosen, to study their regulatory influence on the generation of cytotoxic T lymphocytes *in vitro*. CTLL 1E3, maintained and expanded in the presence of stimulator cells and Con A-SN, showed high H-Y-specific activities as well as lectin-facilitated cytotoxic activities. CTLL 2A5 and 2C5, cultured under similar conditions, had, to varying

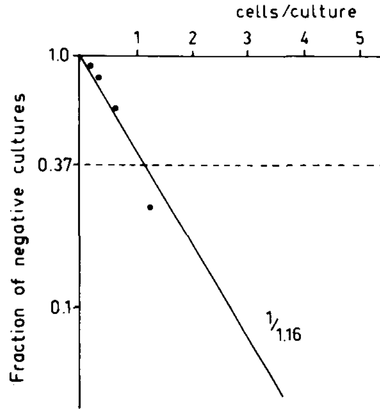

FIG. 1. Limiting dilution analysis of cells killing male B6 target cells in clone 3E6. CTLL 3E6 cells were cultured (24 wells/cell concentration) in 20% Con A-SN in the presence of irradiated B6 spleen cells for 7 days and restimulated for additional 6 days under similar conditions. Thereafter, cultures were assayed for cytotoxicity on ^{51}Cr-labeled male B6 target cells. Cultures were considered positive when lysis exceeded the mean plus 3 SD of 24 control wells containing all ingredients except of clone 3E6 cells. Data are plotted according to Poisson statistics. Minimal estimate of the frequency of CTLP was done as described under Materials and Methods.

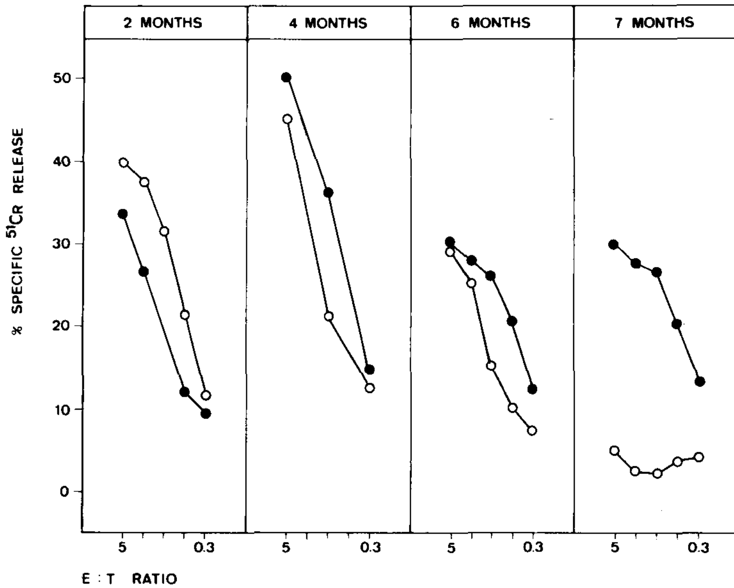

FIG. 2. Comparison between specific cytotoxic activities in long-term cultures of a H-Y-specific T-cell line and a H-Y-specific T-cell clone. The H-Y-specific T-cell line and the H-Y-specific T-cell clone 3E6 were tested at the indicated times in a 4-hr ^{51}Cr-release cytotoxic assay at various effector to target ratios for their ability to lyse ^{51}Cr-labeled male B6 target cells. ●, H-Y-specific cytotoxic T-cell line; O, H-Y-specific T-cell clone 3E6. Numbers on the abscissa indicate effector to target ratio. Percentage specific lysis was calculated as described under Materials and Methods.

TABLE 1

Growth Conditions and Cytolytic Activities of H-Y-Specific T-Cell Clones

H-Y-specific T-cell clone	Growth condition	CTL activity	
		B6	P815 + PHA
1E3	Ag + Con A-SN	+++	+++
2C5	Ag + Con A-SN	++	+++
2A5	Ag + Con A-SN	+	+++
3E6	Con A-SN	±	+++

degrees, lost their specific cytolytic activity, but not their lytic potential as tested on P815 target cells in the presence of PHA. CTLL 3E6, a subline derived from the original cytotoxic clone 3E6 (after 4 months) and adjusted to grow in ConA-SN in the absence of stimulator cells, lysed male B6 target cells only marginally but was still capable of lectin-facilitated cytotoxicity similar to that of CTLL 1E3, 2A5, and 2C5.

Suppressor Activities of CTLL

To investigate their immunoregulatory functions, CTLL cells were irradiated (1200 rad) and added to cultures of female B6 splenic responder cells from primed mice and irradiated (2200 rad) male B6 stimulator cells. After 5 days of incubation, the CTL generated in these cultures were tested on male B6 target cells. Figure 3 shows the results of one representative experiment revealing the different effects of individual CTLL cells on H-Y-specific cytotoxic responses. The data presented in Fig. 3a show that the H-Y-specific CTL responses generated in control cultures (panel 1) were totally abolished by the addition on Day 0 of cells of either CTLL 1E3 (panel 2) or CTLL 3E6 (panel 5) but were only marginally reduced by lymphocytes of CTLL 2A5 and 2C5 (panels 3, 4). These results cannot be explained by assuming that the suppression of CTL generation was in all cases due to the elimination and/or inactivation of stimulator cells in the MLC, because CTLL 2C5 was strongly cytotoxic (Fig. 3d) and poorly suppressive whereas CTLL 3E6 was poorly cytotoxic (Fig. 3d) and strongly suppressive. Another mechanism of suppression is also suggested by the data depicted in Fig. 3b. Here irradiated T lymphocytes of the individual CTLL were admixed to the ongoing MLC on Day 1 rather than on Day 0. Under these conditions only cells of the TCGF-dependent line CTLL (3E6; Fig. 3b, panel 5), but not those of CTLL 1E3, 2A5, or 2C5 (Fig. 3b, panels 2–4), were able to suppress the generation of H-Y-specific CTL.

On the basis of these results it was possible that cells of CTLL 1E3 and 3E6 suppressed the generation of CTL by two different mechanisms. One of these mechanisms (elicited by CTLL 1E3) may be lympholysis of male B6 stimulator cells by the H-Y-specific clone and may be operative only on Day 0 of culture but not on Day 1 after initial activation of precursor cells has taken place. In contrast, cells of CTLL 3E6 may suppress by consumption of TCGF endogenously produced in the MLC, and this mechanism may be effective both on Days 0 and 1.

In order to test this second possibility, exogenous TCGF (MLC-SN) was added to

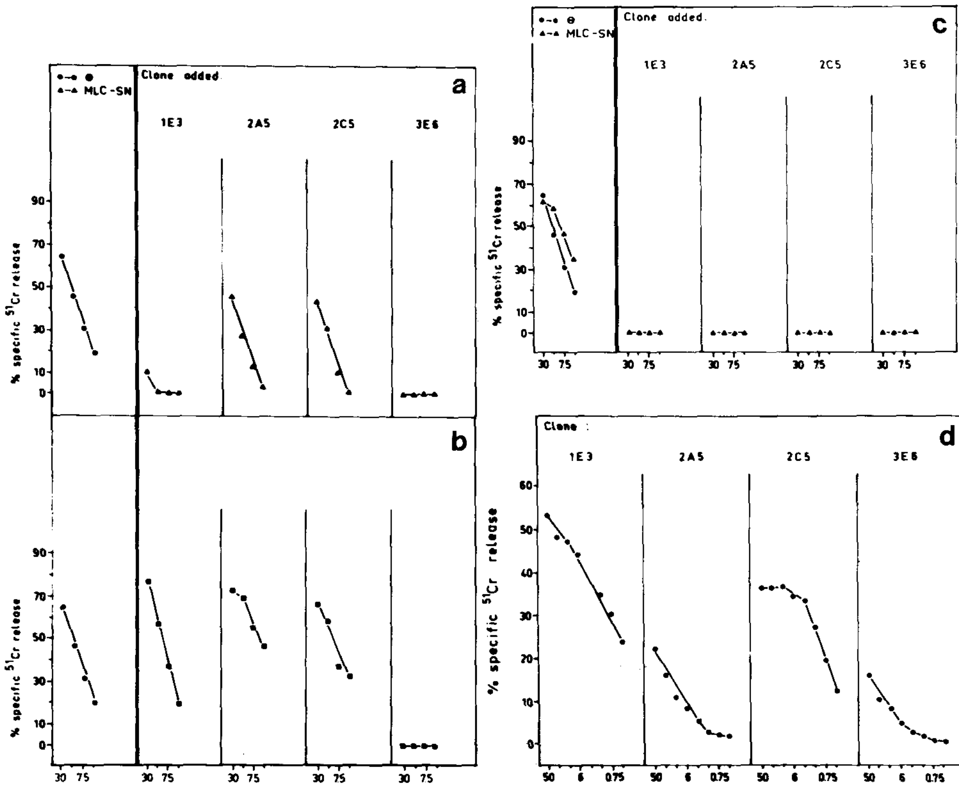

FIG. 3. Suppressive activities (a–c) and cytotoxic activities (d) of H-Y-specific cytotoxic T-cell clones. (a–c) All cultures contained 3×10^6 female B6 responder cells and 5×10^6 irradiated male B6 stimulator cells. Cultures were supplemented either (a) on Day 0 with 1×10^6 irradiated cells of CTLL 1E3, 2A5, 2C5, or 3E6, (b) on Day 1 of culture with the same number of irradiated cloned T cells as a, or (c) on Day 0 with 1×10^6 irradiated cells of CTLL 1E3, 2A5, 2C5, and 3E6 in the presence of MLC-SN (20%). Control cultures were incubated either in the absence (a, b, panel 1) or in the presence (c, panel 1) of MLC-SN. After 5 days of incubation cytotoxic effector activities were tested on ^{51}Cr-labeled male B6 target cells. Numbers on the abscissa indicate the number of female B6 responder cells ($\times 10^4$) cultured on Day 0, the descendants of which are tested on Day 5. Percentage specific lysis was determined as described under Materials and Methods. (d) Serial dilutions of the indicated H-Y-specific cytotoxic T-cell clones (1E3, 2A5, 2C5, 3E6) were tested in a 4-hr ^{51}Cr-release cytotoxic assay on male B6 target cells. Numbers on the abscissa indicate the effector to target ratio. Percentage specific lysis was calculated as described under Materials and Methods.

cultures supplemented on Day 0 with irradiated cells of either CTLL. As shown in Fig. 3c (panel 1), the addition of MLC-SN to the control MLC resulted in an increase (about twofold) of the H-Y-specific CTL response when compared to control cultures incubated without additional MLC-SN. Unexpectedly the addition of MLC-SN to cultures containing irradiated cells of either CTLL resulted in a total abrogation of CTL responses, irrespective of the type of CTLL added. Thus, in no case could the suppression be reversed by the lymphokine source. Moreover, T-cell clones of CTLL 2A5 and 2C5 which only slightly suppressed the generation of H-Y-specific CTL in the absence of MLC-SN (Fig. 3a) became strongly suppressive in its presence. The

experiment in Fig. 4 shows that enhancement of suppression by MLC-SN was not restricted to CTLL 2A5 and 2C5. In this experiment, graded cell numbers of any of the CTLL were titrated into cultures of female B6 responder and male B6 stimulator cells on Day 0. The suppression of H-Y-specific CTL responses was most pronounced at high concentrations (1×10^6/culture) of CTLL added and decreased with decreasing numbers (5×10^5, or 1×10^5/culture) of CTLL lymphocytes added to the cultures. When in addition to CTLL MLC-SN was admixed to the MLC, suppression of H-Y-specific CTL generation was consistently more pronounced with 1×10^6 and 5×10^5 CTLL cells added. These results show that the suppressive capacity of all CTLL can be enhanced by their interaction with lymphokines. Together with our finding that cells of all four CTLL absorbed similar quantities of TCGF from ConA-SN-conditioned medium (see Fig. 5), these results did not favor the view that the consumption of TCGF by CTLL was a major reason for their suppressive activities. It is more likely that the interaction of T-cell clones with MLC-SN results in the induction of yet another suppressive mechanism.

Antigen Specificity of Suppressor Activities of CTLL

MLC were set up consisting of female B6 responder cells and either male B6 spleen cells, TNP-modified female B6 spleen cells, a mixture of both, or male B6 spleen cells modified with TNBS, as stimulators. On Day 0, irradiated cells (1×10^6) of CTLL 1E3 or 3E6 were added to the cultures and the cytolytic activity in each culture well was tested on Day 5, on both male B6 target cells and TNP-modified EL4 tumor

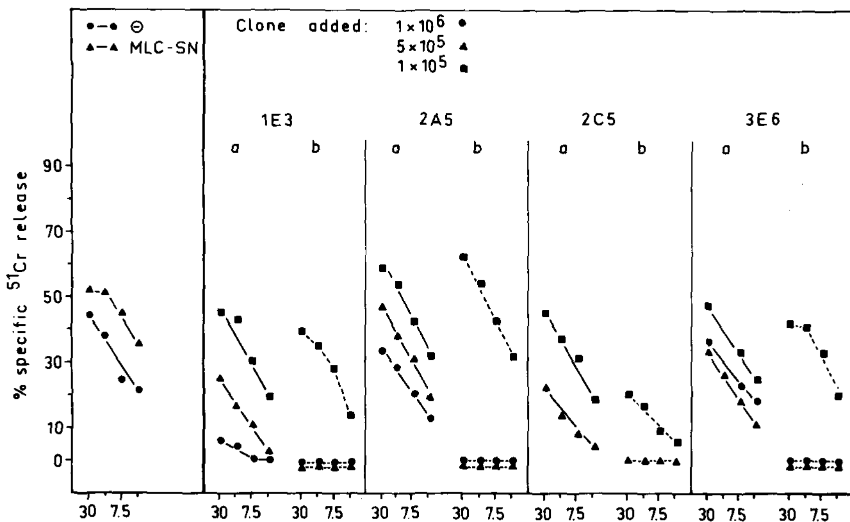

FIG. 4. Suppressive activities of CTLL on the generation of anti-H-Y-specific CTL in the absence or presence of MLC-SN. Cultures containing 3×10^6 female B6 responder cells and 5×10^6 irradiated male B6 stimulator cells received either 1×10^6 (●), 5×10^5 (▲), or 1×10^5 (■) irradiated cells of CTLL 1E3, 2A5, 2C5, or 3E6 in either the (a) absence or (b) presence of MLC-SN (20%). Control cultures (panel 1) were incubated either alone (●) or in the presence (▲) of MLC-SN (20%). After 5 days of incubation effector activities were tested on ^{51}Cr-labeled male B6 target cells. Numbers on the abscissa indicate the number of female B6 responder cells ($\times 10^4$) cultured on Day 0, the descendants of which are tested on Day 5. Percentage specific lysis was calculated as described under Materials and Methods.

Clone	Ag	MLC-SN	t(h)	Residual TCGF Activity (cpm $\times 10^{-3}$) 1 3 5 10
-	+	+	24	
1E3	+	+	24	
2A5	+	+	24	
2C5	+	+	24	
3E6	+	+	24	

FIG. 5. Absorption of TCGF by CTLL. Irradiated cloned T cells (1×10^6) were cultured with irradiated male B6 stimulator cells (5×10^6) in the presence of MLC-SN (20%). After 24 hr the supernatants were harvested and tested for residual TCGF activities on the TCGF-dependent line 96. On Day 3 [^{3}H]TdR was added during the last 4 hr. Specific [^{3}H]TdR was determined by subtracting background responses of line 96 incubated in medium alone.

cells. The data in Fig. 6 show the cytolytic activities of control cultures containing either male B6 (panel 1) or TNP-modified female B6 (panel 2) spleen cells as stimulators on the corresponding target populations. The generation of CTL was totally abrogated by the addition of cells of either CTLL 1E3 or 3E6 in all cultures, irrespective of whether male B6 stimulator cells were present or not (panels 3–6). Additional experiments of the same design revealed that suppressive activities of CTLL could be

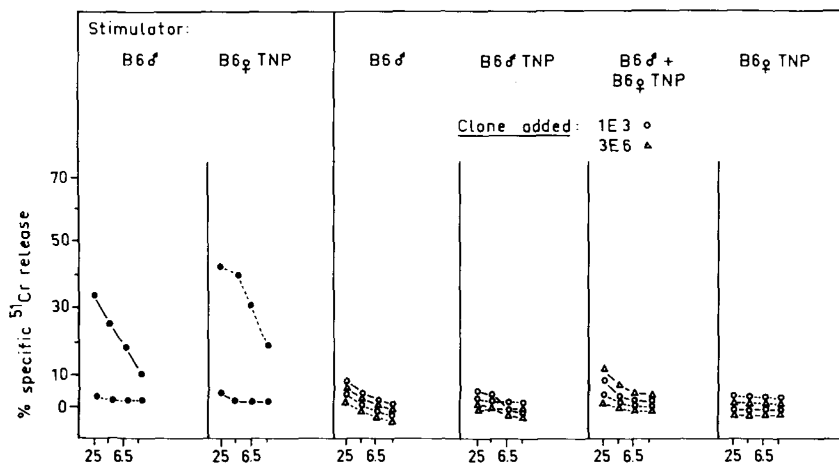

FIG. 6. Antigen specificity of suppressor activities of H-Y-specific cytotoxic T-cell clones. All cultures received 3×10^6 female B6 responder cells and either 5×10^6 irradiated male B6 spleen cells (unmodified, panels 1, 3; or modified with TNP, panel 4), female B6 spleen cells modified with TNBS (panels 2, 6), or a mixture of normal male B6 spleen cells and female B6 spleen cells modified with TNBS (panel 5). Individual cultures received 1×10^6 irradiated cells of CTLL 1E3 (○) or 3E6 (△) (panels 3–6). Control cultures were incubated in the absence of CTLL (panels 1, 2). After 5 days of incubation effector activities were tested on ^{51}Cr-labeled male B6 target cells (——) as well as on TNP-modified EL-4 tumor target cells (- - -). Numbers on the abscissa indicate the numbers of female B6 responder cells ($\times 10^4$) cultured on Day 0, the descendants of which are tested on Day 5. Percentage specific lysis was calculated as described under Materials and Methods.

diluted out as in the experiment in Fig. 4 by reducing the number of CTLL cells added, but suppression was restored in the presence of MLC-SN (data not shown). Similarly, in allogeneic MLC consisting of BALB/c responder lymphocytes and either male B6 or female B6 stimulator cells the addition of irradiated cells of CTLL 3E6 resulted in a nearly total suppression of the anti-H-2^b responses in both cultures (Fig. 7, panel 1 vs panel 3). Strongly suppressed alloreactive CTL responses were observed also in cultures receiving CTLL 3E6 lymphocytes and MLC-SN (Fig. 7, panel 4) which by itself considerably augmented the anti-H-2^b responses in MLC in the absence of cloned T cells (Fig. 7, panel 2). Thus, the data presented here suggested that T-cell clones did not exhibit their suppressive activities in an antigen-specific manner and that at least one of the suppressive mechanisms elicited by CTLL is independent of the recognition of the proper antigens.

Augmenting Activities of CTLL

In the course of this study we had observed that the addition of low numbers (1×10^5) of CTLL to the mixed-lymphocyte cultures on Day 0 often resulted in an increase (two- to fourfold), rather than a decrease, of cytolytic activities when compared to control cultures (see Fig. 4). Further experiments of the same design revealed that

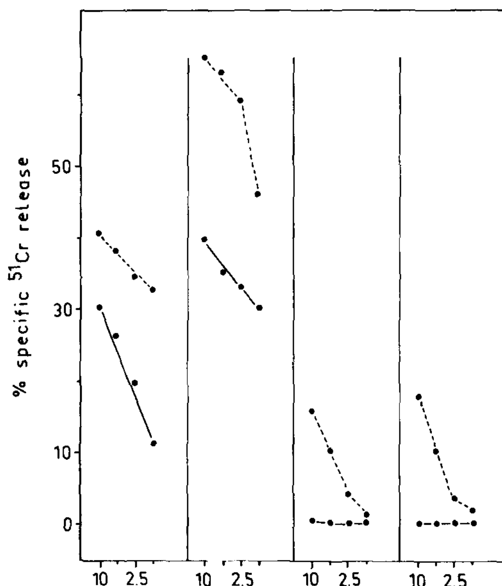

FIG. 7. Suppressive activity of CTLL 3E6 on the generation of a primary anti-H-2^b CTL response. Primary cultures contained 1×10^6 BALB/c responder cells and either 5×10^6 irradiated male B6 spleen cells (——) or 5×10^6 female B6 spleen cells (- - -) as stimulators. Cultures were incubated either alone (panel 1), in the presence of MLC-SN (10%, panel 2), in the presence of 4×10^5 irradiated cells of CTLL 3E6 (panel 3), or in the presence of 4×10^5 irradiated cells of CTLL 3E6 and MLC-SN (10%, panel 4). After 5 days of incubation effector activities were tested on ^{51}Cr-labeled EL-4 tumor target cells. Numbers on the abscissa indicate the number of BALB/c responder cells ($\times 10^4$) cultured on Day 0, the descendants of which are tested on Day 5. Percentage specific lysis was calculated as described under Materials and Methods.

all CTLL and even lower numbers of cloned T cells (down to 1×10^4) were able to significantly enhance cytolytic activities generated in MLC (data not shown). We also tested whether the augmenting activity observed with low numbers of cells of CTLL could be mediated by soluble factors. Irradiated cells of CTLL 1E3, 2A5, 2C5, and 3E6 were cultured in the presence of male B6 stimulator cells for 1 day. Supernatants of the individual cultures were removed and tested either for TCGF activities on a growth factor-dependent cell line or for helper activities in an MLC consisting of female B6 spleen responder cells and male B6 stimulator cells. The data depicted in

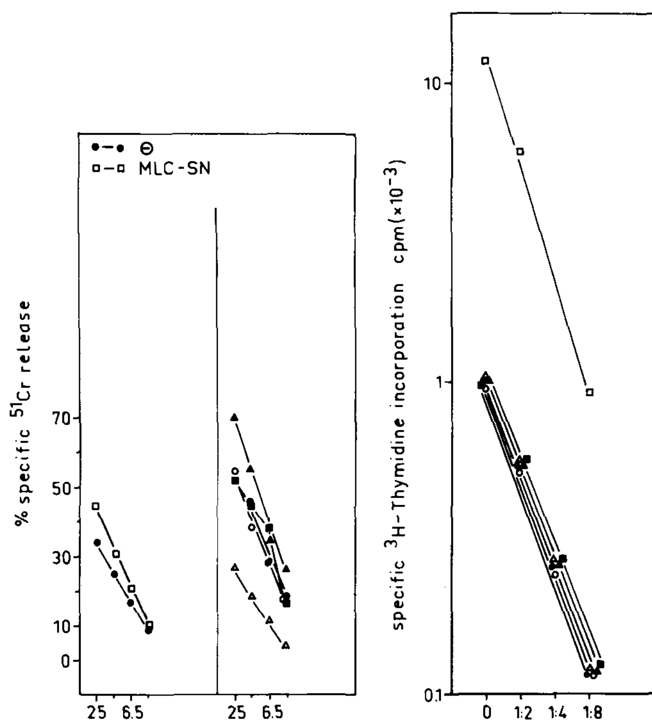

FIG. 8. Lymphokine activities in supernatant of cultures of CTLL stimulated with antigen. Irradiated T cells (1×10^6) of CTLL 1E3 (■), 2A5 (●), 2C5 (▲), and 3E6 (O) were incubated with irradiated and T-cell-depleted male B6 stimulator cells (5×10^6). After 24 hr the supernatants were harvested and tested either for their augmenting activities in an anti-H-Y-specific CTL response (panels 1, 2) or for their TCGF activity on the TCGF-dependent line 96 (panel 3). Female B6 responder cells (3×10^6) and irradiated male B6 stimulator cells (5×10^6) were incubated either in the absence (●) or presence (□) of MLC supernatant (20%) (panel 1) or in the presence of supernatants (50%) derived from cultures of CTLL 1E3 (■), 2A5 (●), 2C5 (▲), or 3E6 (O) or of control supernatant derived from cultures containing only stimulator cells (△) (panel 2). After 5 days of incubation effector activities were tested on ^{51}Cr-labeled male B6 target cells. Numbers on the abscissa indicate the number of female responder cells ($\times 10^4$) cultured on Day 0, the descendants of which are tested on Day 5. Percentage specific lysis was calculated as described under Materials and Methods. For the determination of TCGF activity a fixed number of cells of the TCGF-dependent line clone 96 were incubated with serial dilutions (dilution 0, 50%) of the individual supernatants of CTLL 1E3 (■), 2A5 (●), 2C5 (▲), and 3E6 (O), of control supernatant derived from cultures containing stimulator cells only (△), or of MLC supernatant (dilution 0 = 20%, □). After 3 days [^{3}H]TdR was added and after additional 4 hr, [^{3}H]TdR incorporation was assessed. Specific [^{3}H]TdR incorporation was determined by subtracting background responses of TCGF-dependent T cells incubated in complete medium.

Fig. 8 (panel 3) demonstrate that the background level of TCGF detected in control cultures consisting of stimulator cells only was not increased by the addition of either CTLL. Nevertheless all supernatants derived from cultures containing antigen and cloned T cells, but not those without CTLL, considerably enhanced (two- to fourfold) the generation of H-Y-specific CTL when added on Day 0 to the MLC (Fig. 8; panel 2 vs panel 1). Thus, the data indicate that cloned cells of CTLL 1E3, 2A5, 2C5, and 3E6 are able to amplify, although to varying degrees, the development of CTL from their precursors and suggest that this activity is mediated by soluble factors which are distinct from those reactive with TCGF-dependent lines.

DISCUSSION

The data presented here demonstrate that cloned H-Y-specific cytotoxic T cells and their long-term culture derivatives are able to suppress the generation of CTL *in vitro*. In addition, the experiments show that the same cloned T-cell lines can amplify cytotoxic activities developing in fresh mixed-lymphocyte cultures.

All H-Y-reactive CTLL studied for their regulatory capacities were Lyt-$1^{-}2^{+}$, H-$2D^{b}$ restricted and efficiently lysed male B6 but not irrelevant target cells at low effector cell concentrations at early stages of their *in vitro* cultures. A particular feature of the CTLL was the gradual loss of specific cytolytic activity but not of the lytic potential, as tested in lectin-mediated lympholysis, with time in culture (30). It is not clear at the moment whether this decline in specific cytolytic function is occurring at the same time and at a comparable rate in all cloned T-cell lines.

Four H-Y-reactive CTLL, all of which were derived from one long-term cultured cytotoxic T-cell line, with different growth and functional characteristics were tested for their capacity to regulate the generation of H-Y-specific CTL *in vitro*. Three of them were grown in the presence of antigen in Con A-SN-conditioned medium and showed either high (1E3), intermediate (2C5), or weak (2A5) cytotoxic activity on B6 male target cells. The fourth T-cell line (3E6) was maintained only in the presence of Con A-SN and had lost most of its specific cytolytic activity.

Of these four lines, CTLL 1E3 and 3E6, but not CTLL 2A5 and 2C5, suppressed the development of a secondary anti-H-Y response *in vitro* when added at the beginning of culture. Previous studies on the regulation of alloreactive (4–6, 8, 10, 13, 15, 17) and of H-2-restricted CTL responses (anti-TNP, anti-H-Y, M. M. Simon, unpublished results; anti-minor H (3)) have shown that antigen-activated and irradiated lymphocytes taken from a primary or secondary MLC can inhibit the CTL response of a fresh spleen cell suspension to the same antigen. It was concluded by one group of authors (4, 5, 15) that this suppression involved inactivation of stimulating cells in the second culture by CTL from the primary MLC. In contrast, several other groups (6, 7, 8, 13, 17) concluded that a distinct subset of suppressor cells was responsible for the inhibitory activity. Our data obtained with the four CTLL suggest that yet another suppressive mechanism is responsible for all of our results or that, in addition to stimulator cell killing, additional mechanisms must exist for the inhibition of CTL responses by CTLL clones.

One possibility for a second mechanism was that the suppression observed with CTLL 3E6 was due to the consumption by the cloned T cells of TCGF, which is required for optimal clonal expansion of CTLP in fresh MLC and which is endogenously produced in a secondary anti-H-Y response. Such a mechanism has been

proposed by several authors for the regulatory capacities of mitogen (26), alloantigen-activated lymphocytes (18, 19), or cells from a CTLL (18). Our experiments which directly addressed this question revealed that the addition of MLC-SN together with either CTLL to fresh MLC resulted in total abrogation rather than in a rescue of the anti-H-Y responses (Fig. 3c). The most intriguing aspect of these experiments was that even cells of CTLL 2A5 and 2C5, which by themselves were not able to suppress anti-H-Y specific CTL responses, became strongly suppressive in the presence of MLC-SN. The possibility that this effect was due to a recovery of specific cytolytic activity in noncytolytic CTLL by interaction of T cells with lymphokines was excluded. These findings strongly argue against the consumption of TCGF as the main reason for the inhibition of CTL responses by CTLL. This conclusion is also supported by two additional findings. First, cells of all four CTLL absorbed similar quantities of TCGF from MLC-SN (Fig. 5) but only cells of CTLL 1E3 and 3E6 suppressed the secondary anti-H-Y responses when added at Day 0 of culture (Fig. 3a). Second, when limiting numbers of cloned H-Y-reactive T cells were titrated to fresh MLC in the presence of MLC-SN, total inhibition of anti-H-Y-specific CTL responses was obtained even with cell concentrations which by themselves were not or only marginally suppressive (Fig. 4).

The data therefore suggest that the binding of lymphokines to cloned T cells leads to the induction of suppressive signal(s) which can inhibit the generation of CTL from their precursor cells. In recent experiments we have found that supernatants from mitogen-activated CTLL contain soluble mediators that can suppress the activation and expansion of CTLP in a similar way as the CTLL themselves. We are presently investigating whether all or part of the suppressive activities of CTLL is due to the secretion of such factors (M. M. Simon, manuscript in preparation).

Günther *et al.* (18) and Susskind *et al.* (19) reported that Day 3 MLC activated lymphocytes or a hapten-specific CTL clone which continuously grew in TCGF (18) have suppressive activities in fresh MLC which could be reversed by the addition of TCGF. The apparent discrepancy to our findings may be explained by the distinct lymphocyte populations used to inhibit CTL responses and/or by the different TCGF sources used in both studies. In addition different CTLL may have differential capacities to elicit regulatory functions in response to the lymphokines. It is also possible that these authors may have worked with limiting concentrations of TCGF.

Different regulatory mechanisms for different CTLL are also suggested by our finding that, of the four T-cell clones studied, only CTLL 3E6, but not CTLL 1E3, 2A5, or 2C5, can inhibit cytotoxic responses *in vitro* when added on Day 1 instead of Day 0 to an ongoing MLC (Fig. 3b). A time-dependent susceptibility of alloantigen-activated MLC to suppression has also been observed for presensitized lymphocyte populations (3, 8; M. M. Simon, unpublished results) which were shown to inhibit the generation of CTL only at an early stage (up to 2 days in culture) of culture. These results suggested that some preactivated lymphocytes can interfere with CTL development during the activation, but not during the expansion, phase of antigen-reactive T cells. Taken together our results suggest that individual T-cell lines may interfere with the development of CTL at different stages by virtue of distinct regulatory mechanisms.

In many previous studies both antigen-specific (4, 5, 8, 10, 13, 15, 17) and nonspecific (18, 19; M. M. Simon, unpublished results) suppressor activities were identified in MLC-activated and radioresistant lymphocyte populations. Antigen-specific inhibition

of CTL generation in fresh MLC is observed after addition of low numbers of lymphocytes preactivated to the relevant alloantigens whereas high numbers of MLC-activated cells were required for the detection of nonspecific suppression activity (M. M. Simon, unpublished). It was not clear from these studies whether both activities were elicited by only one or by different T-cell subsets. Although the cytolytic activities elicited by all our H-Y-reactive T-cell lines originally were strictly specific for male B6 target cells, none of the CTLL exhibited a similarly exclusive specificity when tested for their suppressive capacity. Since interaction of cells from all CTLL with MLC-SN alone resulted in the development of strong suppressive activities, it is possible that the presence of lymphokines in test cultures may prevent the detection of antigen-specific suppression. However, the results demonstrate that the presence of the appropriate antigen is not required in cultures of CTLL for suppression to become effective and suggest that at least some suppressive interactions of CTL are antigen independent.

All of our CTLL enhanced cytotoxic activities in fresh MLC when admixed at low cell numbers (Fig. 4). This effect could be transferred by supernatants from CTLL cultures presensitized to male B6 stimulator cells (Fig. 8). The soluble product was distinct from TCGF and possibly also from the CTL differentiation factor (CDF) (32, 33) since it could not provide the signal for the differentiation of CTL from their precursors in thymocyte populations (data not shown). Therefore other lymphokines with amplifying activity must be secreted by the H-Y-reactive CTLL.

Taken together, these data provide evidence for multiple roles of cytotoxic effector cells in the regulation of maturation and clonal expansion of their precursor cells. We do not know, at present, whether any of the described effects plays a role in the generation and regulation of cytotoxicity *in vivo*. We consider the possibility, however, that cytotoxic T-cell clones and their noncytolytic derivatives may represent different functional stages in the development of CTL effector cells *in vivo* which participate directly or indirectly in the generation of effective cytotoxic immunity. Furthermore, cytotoxic T-cell clones can act as T suppressor cells in the CTL system and perform an additional regulatory function, i.e., amplification. These observations put considerable constraints on the view that each immunological function is the expression of one particular subclass of lymphocytes.

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