HELPER CELLS IN THE AUTOLOGOUS MIXED LYMPHOCYTE REACTION

III. Production of Helper Factor(s) Distinct from Interleukin 2*

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Activation of cytotoxic T lymphocytes (CTL)[1] in vitro in response to antigen requires a complex series of interactions among T cell populations and macrophages. The demonstration that soluble factors produced by the various cell types involved can mediate these interactions (1, 2) has facilitated the identification of this pathway of T cell activation.

Two cytokines, interleukin 1 (IL-1) and interleukin 2 (IL-2), are important in the development of cytotoxic potential. In the presence of antigen, macrophages are induced to produce IL-1 (3). The IL-1 provides a signal for a population of helper/inducer T cells to synthesize IL-2 (4). The released IL-2 binds to receptors on antigen-sensitized cytotoxic T cell precursors and leads to the differentiation of antigen-specific cytotoxic effector cells (5), possibly by stimulating the production of immune interferon (6) by Lyt-1^-23^+ T cells. In the absence of T helper cells exogenous IL-2 can directly activate cytotoxic precursors against specific antigenic stimuli (7, 8).

Much of our recent work has focused on the function of murine T cell populations involved in the response to syngeneic I-region determinants in the autologous mixed lymphocyte reaction (AMLR) (9, 10). The responding cells in the AMLR are Lyt-1^+23^- T cells, suggesting that they are in the helper-inducer class (11, 12). In previous papers (9, 13), we have demonstrated that T cell recognition in the AMLR can activate helper cells that, in the presence of hapten-modified determinants on syngeneic, nonstimulatory cells, can induce the generation of H-2-restricted, hapten-specific cytotoxic activity. Similar results have also been described in humans (14, 15). The studies in the human system also described a factor obtainable from AMLR cultures that facilitated the cytotoxic T cell response to nonstimulatory (heat-treated) allogeneic cells or to 2,4,6-trinitophenyl- (TNP) modified autologous peripheral blood lymphocytes (14, 15). It was suggested that this factor might be IL-2 (14). Recent reports have demonstrated the presence of IL-2 in AMLR supernatants from both human (16) and mouse (17, 18) cultures. This was established by the ability of these supernatants to promote the continuation of a mitogen-activated proliferative response

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[1] *Abbreviations used in this paper*: alpha-MM, alpha-methyl-D-mannopyranoside; AMLR, autologous mixed lymphocyte reaction; C, complement; Con A, concanavalin A; CSF, colony-stimulating factor; CTL, cytotoxic T lymphocytes; FCS, fetal calf serum; IL-1, interleukin 1; IL-2, interleukin 2; IL-3, interleukin 3; LU, lytic units; NA(thy), nylon wool column-nonadherent thymocytes; PHA, phytohemagglutinin; TCGF, T cell growth factor; TNP, 2,4,6-trinitrophenyl.

or to maintain an IL-2-dependent cell line. In the mouse, Lyt-1^+23^- T cells are involved in the response in the AMLR and in the production of IL-2.

The purpose of this study is to explore the role of murine AMLR culture supernatants in the generation of cytotoxicity to modified self and to define the relationship of the factor(s) present in the supernatants to known cytokines. Evidence will be presented to show that the factor(s) we have obtained from our AMLR cultures is distinct from IL-2 and IL-1 and is made by Lyt-1^+23^- T cells. The position and the role of the AMLR supernatant factor in the cascade of cellular interactions leading to the activation of antigen-specific, H-2-restricted CTL will be discussed.

Materials and Methods

Mice. BALB/c, C3H, C57BL/6, and DBA/2 mice were purchased from the Laboratory Animal Facility of the Institute for Cancer Research, Philadelphia, PA.

Cell Preparation and Culture Medium. Mice were killed by cervical dislocation, the spleens or thymuses were asceptically removed, and single cell suspensions were prepared as previously described (19). T cells were obtained by recovering nonadherent cells from twice-nylon wool-passed spleen cells (20). Where indicated, thymus cell suspensions were depleted of nylon wool-adherent cells by the same procedure. The culture medium was RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum (FCS; Reheis Chemical Co., Phoenix, AZ), 5×10^{-5} M 2-mercaptoethanol, 20 mM Hepes buffer, 100 U/ml penicillin, and 100 μg/ml streptomycin. Batches of FCS were screened, and those that produced the lowest background proliferative responses were selected.

AMLR Supernatants. Cultures of 5×10^6 T cells plus 5×10^6 mitomycin-C-treated (25 μg/10^7 cells for 30 min at 37°C) syngeneic spleen cells (AMLR), T cells alone, mitomycin-C-treated spleen cells alone, or Lyt-1^+-enriched T cells plus mitomycin-C-treated syngeneic spleen cells were incubated in 24-well flat-bottomed culture plates (3524; Costar, Data Packaging, Cambridge, MA) in a final vol of 2.0 ml at 37°C in 95% air, 5% CO_2 for 2 or 3 d, as indicated. Supernatants were recovered, centrifuged at 1,000 *g* for 10 min, passed through 0.45-μm filters (Millipore Corp., Bedford, MA), and frozen until ready for use.

T Cell Growth Factor (TCGF) Preparation. IL-2-containing conditioned medium (TCGF) was prepared from concanavalin A- (Con A) stimulated spleen cells as previously described (21). When TCGF was used in the cytotoxic assay all cultures contained alpha-methyl-D-mannopyranoside (alpha-MM; Sigma Chemical Co., St. Louis, MO) at a final concentration of 0.05 M.

Antibody Treatments. T cells were depleted of Lyt-123^+ and Lyt-1^-23^+ cells using an anti-Lyt-2 hybridoma (22), clone 53-6.7, generously given to us by Dr. D. E. Mosier, Institute for Cancer Research. T cells at 10^7/ml were treated with the antibody as undiluted culture supernatant according to published methods (18).

AMLR cultures were depleted of T cells or non-T cells with monoclonal anti-Thy-1.2 antibody or monoclonal anti-Ia^d antibody as culture supernatants derived from hybridomas 3X3 (gift of Dr. J. Sprent, University of Pennsylvania, Philadelphia, PA) and MKD6 (developed by P. Marrack and J. Kappler; a gift of Dr. D. E. Mosier), respectively. AMLR cultures were treated with 1 ml of a 1:10 dilution of anti-Thy-1.2 or undiluted anti-Ia^d per 10^7 cells for 30 min at 37°C, washed twice, and treated with guinea pig complement (C) for 30 min at 37°C. The antibody-treated AMLR cultures and control cultures treated only with culture medium and complement were layered over a 40% Percoll gradient (Pharmacia, Fine Chemicals, Div. Pharmacia, Inc., Piscataway, NJ) and centrifuged at 1,000 *g* for 10 min. By this method viable cells are pelleted and dead cells remain at the surface. The pelleted cells were washed three times and recultured at the original volume for an additional 2 d, at which time supernatants were prepared as described above.

Assay for Help of Cytotoxic Cell Activation. Cultures of 4×10^6 T cells alone, or with 4×10^6 mitomycin-C-treated syngeneic spleen cells and 4×10^6 TNP-modified, mitomycin C-treated syngeneic thymocytes were done in 24-well flat-bottomed plates. The procedure for hapten modification has been previously described (23). To test for helper factors, supernatants from AMLR and control cultures, or TCGF in some experiments, were added to the assay system at

the indicated concentrations in place of the syngeneic spleen cells. The final vol throughout was 2.0 ml.

All cultures were incubated in 95% air, 5% CO_2 at 37°C for 5 d. Replicate wells were then pooled, viability determined, and the cells plated in triplicate in 96-well round-bottomed culture plates (Linbro Chemical Co., Hamden, CT) with 10^4 ^{51}Cr-labeled target cells at final effector/target ratios of 20:1, 10:1, and 5:1 unless otherwise indicated. The methods of performing the assay and calculating specific lysis have been previously described (23). Lytic units were calculated by the method of Cerrotini and Brunner (24). Briefly, cytotoxic activity was plotted on a semilogarithmic scale against numbers of effector cells. Linear regression lines were drawn from cytotoxic values at the multiple effector/target ratios. One lytic unit (LU) was defined as the number of effector cells required to generate 25% cytotoxicity. LU per culture were determined by dividing the number of cells in 1 LU into the number of viable cells recovered from each culture.

In the experiments in which LU were reported against the haptenated targets, only background levels of cytotoxicity were detected against unmodified targets at an effector/target ratio of 20:1.

Assessment of Cytokine Activity. AMLR and TCGF supernatants were tested for their ability to support the growth of an IL-2-dependent cell line and to act as a co-stimulator in the response of thymocytes to Con A and phytohemagglutin (PHA). The IL-2-dependent cell line T22A6 was derived from a C57Bl/6 cytotoxic cell clone and was maintained in TCGF. Both the cell line and the assay for testing for IL-2 activity were given to us by Dr. H. R. Snodgrass (Institute for Cancer Research). To measure IL-2, 2×10^4 cell line cells were cultured with serial dilutions of the test supernatants in 96-well flat-bottomed plates (3596; Costar) in a final vol of 0.1 ml. After 24 h at 37°C individual wells were pulsed for 4 h with 0.5 μCi [^{125}I]-iododeoxyuridine; (New England Nuclear, Boston, MA). Cultures were harvested onto glass fiber filter paper (934 AH; Reeve Angel, Clifton, NJ) with an automated multi-sample harvester (MASH II; Microbiological Associates, Walkersville, MD). Incorporation of radiolabel was measured on a Beckman Biogamma (Beckman Instruments, Inc., Fullerton, CA). The proliferative response was calculated by subtracting the counts per minute in the cell line cultured alone from cultures containing dilutions of the test supernatant. IL-2 activity was calculated as decribed by Farrar et al. (25). The Con A and PHA co-stimulator assays were performed with CBA or BALB/c thymocytes, as reported in the literature (8, 26), and units of activity calculated according to the method of Farrer et al. (25). In our hands the cell line will detect IL-2; the Con A co-stimulator assay primarily measures IL-2, but also detects IL-1 to some extent; and the PHA thymocyte assay detects IL-2 or IL-1 equally well (J. A. Wolos and J. B. Smith, unpublished observations).

Results

Helper Factors Produced in the Murine AMLR Capable of Mediating a Cytotoxic Response. In experiments designed to determine if soluble factors from murine AMLR cultures were capable of mediating a cytotoxic response to modified self, as has been shown in humans (14, 15), BALB/c AMLR culture supernatant from day 3 of culture was added into fresh cultures containing BALB/c T cells and syngeneic TNP-modified, mitomycin-C-treated thymocytes. As seen in Table I, and in agreement with our previous reports (9, 13), the addition of TNP-modified T cells to an AMLR resulted in the recovery of substantial cytotoxic activity against TNP-modified syngeneic targets after 5 d in culture. In the absence of the splenic stimulators virtually no cytotoxicity developed. However, the addition of AMLR supernatants into these cultures resulted in the activation of CTL directed against hapten-modified self targets. The maximum helper activity was demonstrable with day-3 AMLR supernatant at 25% of the final vol. Kinetic studies reveal that helper factors appear to be present in AMLR cultures as early as day 1, with maximum activity obtained by day 3 or 4 (data not shown).

TABLE I

*Effect of AMLR Supernatants on the Generation of Cytotoxicity to TNP-modified Self**

Experiment	Cultures	BALB/c AMLR supernatant (25% vol/vol)	Cytotoxicity; TNP-BALB/c
			LU/culture
1	BALB/c		
	T + $spleen_m$ + TNP-thy_m	—	24.7
	T + TNP-thy_m	—	0.0
	T + TNP-thy_m	10%	6.4
	T + TNP-thy_m	25%	26.8
	T + TNP-thy_m	50%	21.4
2	BALB/c T	+	0
	TNP-thy_m	+	0
	T + TNP-thy_m	+	26.3

* T cells (T), mitomycin-C-treated spleen cells ($spleen_m$), and TNP-modified, mitomycin-C-treated thymocytes (TNP-thy_m) were all at concentrations of all at 4×10^6/well in 2.0-ml vol.

These results do not allow us to determine if both T cells and TNP-modified thymocytes are necessary for the AMLR supernatants to mediate a response. Some mitogens, such as Con-A, will polyclonally activate cytotoxic precursor T cells in the absence of the target antigen (27). If the AMLR factor acts as a T cell mitogen, TNP-self-directed cytotoxicity could occur in the absence of TNP-modified thymocytes. Alternatively, cytokines such as IL-2 have been demonstrated to activate cytotoxic precursor cells in the thymus in the presence of hapten-altered self (7). It is possible that TNP-modified thymocytes act as both the antigen-presenting cells and the source of the cytotoxic effectors. In this study, the addition of day-3 AMLR supernatants to purified T cells at the initiation of culture resulted in no demonstrable cytotoxic activity (Table I, experiment 2). Likewise, TNP-modified, mitomycin-C-treated thymocytes failed to generate TNP-induced self-directed cytotoxic cells when cultured in the presence of AMLR supernatants. Significant cytotoxic activity was detectable only in those cultures that contained T cells, TNP-modified thymocytes, and AMLR supernatants. Thus it appears the cytotoxic activity mediated by AMLR supernatants requires an interaction between T cells and TNP-modified, mitomycin-C-treated thymocytes.

Interacting Cell Types in the Generation of AMLR Supernatant Helper Factors. Our initial experiments determined that AMLR supernatants contained helper factors that could mediate a CTL response. Further work was done to investigate the cellular interactions required for the production of this factor. In experiment 1 of Table II, day-3 supernatants from T cells cultured alone, mitomycin-C-treated spleen cells cultured alone, or T cells plus mitomycin-C-treated spleen cells (AMLR) were assayed for helper activity. The separate responder and stimulator populations did not produce helper factors. Only the AMLR supernatants were active in helping the generation of cytotoxicity against TNP-modified self.

In experiment 2 of Table II, T cell populations that had been depleted of Lyt-123^+

TABLE II
*Cytotoxic Activity of Cultured Cell Supernatants**

Experiment	Cultures	Supernatant source (25% vol/vol)	Cytotoxicity; TNP-BALB/c
			LU/culture
1	BALB/c T + TNP-thy$_m$	—	0
		T	0
		Spleen$_m$	0.2
		T + spleen$_m$ (AMLR)	26.3
2	BALB/c T + TNP-thy$_m$	—	0
		T + spleen$_m$ (AMLR)	28.4
		Lyt-1$^+$T + spleen$_m$	30.3

* See footnotes to Table I.

and Lyt-1$^-$23$^+$ T cells by treating with a monoclonal anti-Lyt 2 serum plus C (Lyt-1$^+$ T cells) were incubated with mitomycin C-treated syngeneic spleen cells for 3 d. Supernatants from these cultures were assayed for helper activity and compared with supernatants from AMLR cultures containing untreated T cells. The cultures containing Lyt-1$^+$ T cells produced as much or more helper factors than the conventional AMLR. This implies a major role for the Lyt-1$^+$ helper/inducer T cell in the production of AMLR helper factors. Cells within The Lyt-1$^+$ T cell population have been reported to be the responder in the AMLR (11, 12).

Cells Responsible for the AMLR Helper Factor. Data in Table II provided evidence that a Lyt-1$^+$23$^-$ T cell-spleen cell interaction was necessary for the production of the AMLR helper factor. It has been mentioned that as early as day 1 of culture, helper factors can be detected in AMLR supernatants. This suggests that the cellular recognition events initiating the production of the factor have taken place by day 1. If that is the case, removal of the cell populations not involved in the actual synthesis of the factor after day 1 might not affect its production. To test this hypothesis BALB/c AMLR cultures were harvested on day 1 and incubated with a monoclonal anti-Thy-1.2 plus C, monoclonal anti-Ia^d plus C, or culture medium plus C. After the antibody treatment dead cells were removed on a 40% Percoll gradient. This treatment resulted in substantial cell loss using either monoclonal antibody. The viable cells were resuspended up to the original volume and cultured for an additional 2 d, at which time supernatants were assayed for helper activity. In Table III it is seen that day-3 supernatants from AMLR cultures treated only with medium and C on day 1 were strongly active in inducing the generation of TNP-modified self-directed cytotoxicity. Treatment of the AMLR cultures on day 1 with anti-Thy-1.2 plus C resulted in the recovery of day-3 supernatants with no significant helper activity. Interestingly, removal of Ia-bearing splenic B cells and macrophages from the AMLR stimulator population with anti-Ia^d plus C on day 1 slightly enhanced the production of helper factors as measured by the activity of day-3 supernatants. These results strongly suggest that the AMLR helper factor is made by a T cell, and combined with the data in experiment 2 of Table II, suggest that the T cell has the Lyt-1$^+$23$^-$ phenotype.

Effect of AMLR Supernatants and TCGF on the Activation of Cytotoxic Precursors in Thymocytes. Numerous cytokines have been demonstrated to function in the genera-

TABLE III

*Effect of Antibody Treatment of the AMLR on the Generation of Helper Factors**

	Day-3 AMLR supernatants‡	Treatment on day 1	Cytotoxicity; TNP-BALB/C
			LU/well
BALB/C			
T + TNP-thy_m	−	—	0.0
T + TNP-thy_m	+	C only	21.3
T + TNP-thy_m	+	anti-Thy 1.2 + C	0.2
T + TNP-thy_m	+	anti-Ia^d + C	39.0

* See footnotes to Table I.
‡ 50% vol/vol in culture.

TABLE IV

*AMLR Factor Is Not IL-2 and Does Not Activate Thymocytes**

BALB/c cultures‡	BALB/C supernatants	Cytotoxicity; TNP-BALB/c
		LU/culture
T + TNP-thy_m	—	0
	25% AMLR	16.8
Thymocytes + TNP-thy_m	—	0
	10% AMLR	0
	25% AMLR	0
	50% AMLR	0
T + TNP-thy_m	25% TCGF	30.5
Thymocytes + TNP-thy_m	10% TCGF	6.1
	25% TCGF	16.9
	50% TCGF	24.0

* See footnotes to Table I.
‡ All cultures have 0.05-M alpha-MM final concentration.

tion of cytotoxicity in vitro (1, 2). One of these, IL-2, is produced by Lyt-1^+ helper/inducer T cells and can activate cytotoxic precursors in thymus, spleen, or lymph node in the presence of an antigenic stimulus such as allogeneic cells or hapten-modified self (2, 7, 8). The requirement for mature helper T cells is bypassed. Recently it has been reported that IL-2 is produced in an AMLR (17, 18). If our cultures are producing IL-2, we would expect to be able to activate thymocytes in this system. In Table IV, the results of adding AMLR supernatants or IL-2-rich, Con A-stimulated spleen cell supernatants (TCGF) to cultures containing T cells or thymocytes as the responding cells are shown. All cultures contained 0.05 M alpha-MM to bind any Con A in the supernatants. When splenic T cells were cultured with TNP-modified thymocytes, both the AMLR supernatants and the TCGF preparations activated cells cytotoxic for hapten-modified self targets. However, only the TCGF preparation induced the maturation of cytotoxic precursors in the thymocyte population. At no

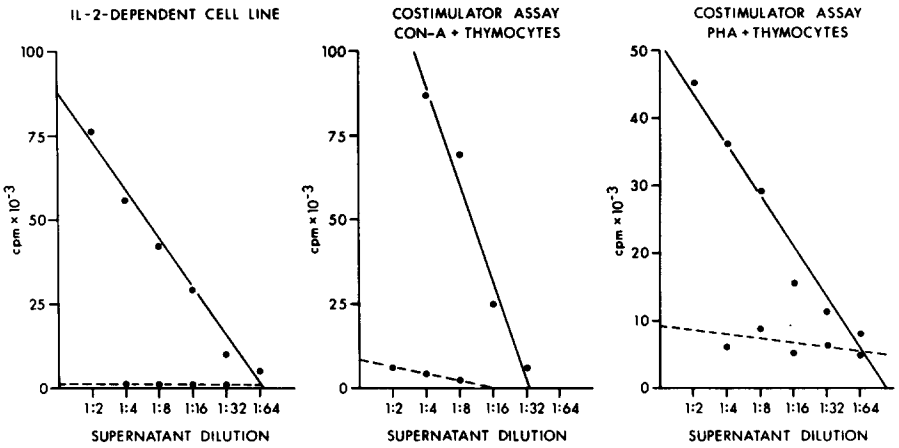

FIG. 1. Function of AMLR supernatants in maintaining an IL-2-dependent cell line or in acting as co-stimulator in response to Con A or PHA. Dilutions of AMLR supernatants (●---●) or IL-2-containing TCGF (●—●) were titrated into cultures and the proliferative response was measured on day 1 (cell line) or day 3 (co-stimulator assay).

concentration tested did the AMLR supernatants activate thymocytotoxic precursors in the presence of hapten-modified self. Thus the factor in the AMLR supernatants does not have one of the biological attributes of IL-2: the ability to activate cytotoxic precursors from the thymocyte population.

Support of an IL-2-dependent Cell Line, or Activity in Co-stimulator Assays, of AMLR Supernatants. The previous experiments suggest the AMLR factor is distinct from IL-2 in that it only affects mature T cells. To further examine this possibility, AMLR supernatants were tested for their ability to support an IL-2-dependent cell line and for their ability to act as a co-stimulator in the response of thymocytes to Con A (8) or to PHA (26). Fig. 1 shows that the IL-2-containing TCGF standard was able to maintain a strong dose-dependent proliferative response by the cell line. The AMLR supernatants, at all concentrations tested, did not support the continued growth of this line. Likewise, although our TCGF preparation was highly active in both the Con A and PHA co-stimulator assays, AMLR supernatants had insignificant activity. These results support the conclusion that the factor or factors present in the AMLR supernatants are not IL-2.

Another cytokine that activates mature helper T cells and induces them to produce IL-2 is IL-1. However, IL-1 is produced by macrophages (4), and we have demonstrated the factor is synthesized by T cells. In addition, the co-stimulator assays will detect IL-1 to varying degrees (25, 26; and J. A. Wolos and J. B. Smith). The inactivity in these assays provides further evidence that the factor in the AMLR supernatants is not IL-1.

Target of AMLR Supernatant Factor. The preceding data demonstrate that the helper factor obtained from the AMLR is synthesized by Lyt-1$^+$ T cells, but is not IL-2. Because T cells produce it, it is also not IL-1. A population of T cells has been postulated that interacts with macrophages to induce them to produce IL-1 (28, 29). It is possible that the factor we are studying is a T cell mediator initiating macrophage activation. Although it would appear there are no macrophages in our cytotoxic assay system, the thymocyte populations have been reported to contain a small number of

macrophages and other cells, such as dendritic cells, which are capable of producing IL-1 (30, 31). These cells cannot present antigen to the T cells in our system in the absence of exogenous helper factors, as evidenced by the consistently poor cytotoxic activity demonstrable in cultures composed of only T cells and TNP-modified, inactivated thymocytes. However, it is possible the addition of AMLR helper factors could induce these cells to make IL-1 and thus initiate the process of cytotoxic cell activation. To examine the role of non-T cells in the thymocyte population in the AMLR-factor-mediated induction of cytotoxicity, thymocytes were depleted of non-T cells by passage over nylon wool columns before TNP modification. The results of using these cells in our cytotoxic assay system are presented in Table V.

In the absence of syngeneic splenic stimulators or AMLR supernatants, no cytotoxicity developed regardless of whether unselected or nonadherent thymocytes were used as the hapten carrier. In agreement with previous experiments, the addition of syngeneic mitomycin-C-treated spleen cells to the cultures of T cells plus hapten-modified thymocytes resulted in the generation of substantial cytotoxic activity against TNP-modified self targets. The use of TNP-modified nylon wool column-nonadherent thymocytes [(NA)thy] had no significant diminishing effect on the generation of cytotoxicity. However, when AMLR supernatants were substituted for the splenic stimulators in this system a disparity between the results obtained using TNP-modified unselected thymocytes or TNP-modified (NA)thy was observed. In those cultures in which the hapten was presented in the context of (NA)thy, the ability of AMLR supernatants to help in the activation of TNP-modified self-specific cytotoxicity was severely depressed compared with similar cultures using unselected thymocytes. These results suggest that the AMLR supernatant mediates its effect through a nylon wool-adherent thymocyte.

Discussion

Our data demonstrate that supernatants from murine AMLR cultures contain a helper factor(s) that aids in the development of hapten-specific cytotoxicity. It has become clear that multiple factors produced by interacting cell types mediate the cytotoxic T cell response. IL-2 (32), a product of activated Lyt-1^+23^- T cells (8), is able to amplify the cytotoxic response of spleen cells or thymocytes to allogeneic

TABLE V

*Loss of Helper Activity in the Absence of Thymic Adherent Cells**

Cultures	BALB/c AMLR supernatants (25% vol/vol)	Cytotoxicity: TNP-BALB/c
		LU/culture
T + $spleen_m$ + TNP-thy_m	−	36.6
T + TNP-thy_m	−	0.0
T + TNP-thy_m	+	20.5
T + $spleen_m$ + TNP-(NA)thy_m	−	29.1
T + TNP-(NA)thy_m	−	0.0
T + TNP-(NA)thy_m	+	0.0

* See footnotes to Table I.

tumor cells and supports generation of cytotoxic activity by spleen cells in response to nonstimulatory ultraviolet-light-treated allogeneic spleen cells (2). IL-2 production requires the presence of macrophages (8). These macrophage functions are mediated by another cytokine, IL-1 that can, like IL-2, be obtained from Con A-stimulated spleen cell cultures (29), but is frequently prepared from activated peritoneal exudate cells or macrophage tumor lines (1, 4, 33). IL-1 can substitute for macrophages in inducing the production of IL-2 (4, 25).

These are the two best-characterized factors in the pathway of cytotoxic T cell activation, with macrophages producing IL-1, which in turn activates appropriate Lyt-1^+23^- T cells to produce IL-2, which provides a proliferation and/or differentiation signal to antigen-sensitized cytotoxic T cell precursors. It is apparent, however, that there are other factors and cell types involved. Reports in the literature have suggested that immune interferon, produced by Lyt-1^-23^+ T cells (34) in response to IL-2, assists in the maturation of CTL (6). Immune interferon can also be found in supernatants from cultures of Con A-stimulated spleen cells (35).

There is recent evidence that one of the early events in the initiation of the CTL response is an interaction between T cells and macrophages resulting in the production of IL-1 (29). The Lyt-1^+23^- T cell involved is distinct from the IL-2-producing T cell and it may function via a soluble mediator, which has been termed interleukin 3 (IL-3) (28). The use of this notation for the factor is not generally accepted, as a number of other reports have described active mediators also designated as IL-3, which may or may not be related to the T cell factor mentioned above (36–38). One better-defined mediator, colony-stimulating factor (CSF), may be similar to the macrophage-inducing IL-3. It is present, as are all the previously mentioned factors, in supernatants from cultures of Con A-stimulated mouse spleen cells (39) and is also produced by Con A or phorbol myristic acetate stimulation of a thymoma cell line, suggesting T cell origin (40, 41). CSF has been demonstrated to activate macrophages to produce IL-1 (42) and therefore may be crucial in the differentiation of cytotoxic precursors.

The autologous mixed lymphocyte reaction (AMLR) measures the proliferative response of T cells to autologous or syngeneic I region determinants (9, 10) and is detectable in both mouse (43) and human (44) systems. Helper cells or uncharacterized factors in human AMLR cultures have been shown to promote a CTL response to non-stimulatory (heat-treated) allogeneic cells or to TNP-modified autologous peripheral blood lymphocytes (14, 15). In addition, helper factors have been obtained from the human AMLR that induced fresh peripheral blood mononuclear cells to produce antibody either in the presence or absence of antigen (15, 45).

We have previously described a method for using helper T cells activated in a murine AMLR to mediate a cytotoxic response to hapten-modified self (9, 13). In the present study, a factor present in AMLR cultures mediated an interaction between T cells and TNP-modified inactivated syngeneic thymocytes in the absence of syngeneic stimulator cells, resulting in the generation of hapten-specific cytotoxic effectors. The production of the helper factor was dependent on an interaction between T cells and mitomycin-C-treated spleen cells. Depleting Lyt-123^+ and Lyt-1^-23^+ T cells before culture in the AMLR had no effect on the production of helper factor. Although these data do not determine whether the factor is being made by Lyt-1^+23^- T cells or by cells in the stimulator population, it proves that Lyt-123^+ and Lyt-1^-23^+ T cells are not necessary for its production.

The identity of the cell type responsible for producing the AMLR helper factor was clarified because as early as day 1, helper activity can be detected in AMLR culture supernatants, indicating the recognition events necessary for factor production had taken place by day 1. The depletion of T or non-T cells in the AMLR on day 1 using monoclonal antibodies, with subsequent reculture until day 3, revealed that active supernatants were recoverable only from the non-T-depleted cultures (T cells). It is interesting to note that the depletion of the Ia^d-bearing stimulator cells after 1 d of culture did not adversely affect the production of the helper factor. It is possible that the anti-Ia^d treatment did not remove all the Ia-positive cells, although similar dilutions of antibody have abolished the proliferative response in both AMLR and mixed lymphocyte cultures (J. A. Wolos and J. B. Smith, unpublished observations). Regardless of whether the stimulating cells were totally removed, the presence of helper factors in AMLR cultures treated with anti-Ia^d and the total absence of helper activity in those cultures treated with anti-Thy-1.2 strongly suggest that the AMLR helper factor is produced by a T cell. These data, combined with the evidence that active factors were recovered from AMLR cultures with Lyt-1^+23^- responder T cells, provide strong evidence that the AMLR factor is produced by a Lyt-1^+23^- T cell.

The similar biological acivities of our AMLR factor and IL-2 preparations in the activation of splenic cytotoxic cells, as well as the dependence on the presence of Lyt-1^+23^- T cells for the production of both factors, suggested our AMLR helper factor might be IL-2. Indeed, culture supernatants recovered from the AMLR in mice (17, 18) and humans (16) have been reported to contain factors with properties of IL-2. Unlike the results reported for IL-2 (27, 28), when our AMLR supernatants were added to cultures of thymocytes plus TNP-modified, inactivated thymocytes, no cytotoxicity developed despite the ability of these same supernatants to activate splenic T cells in response to TNP-modified, inactivated thymocytes. IL-2-containing TCGF from Con A-stimulated spleen cells was able to help both thymocytes and mature T cells in the generation of hapten-specific cytotoxicity. The inactivity of the AMLR supernatants when cultured with immature T cells suggests the factor is not IL-2. Our AMLR helper factors is probably different from the non-IL-2 mediator recoverable from a secondary AMLR (46), as those supernatants will also activate thymocyte precursors for cytotoxic cells. Further support for the conclusion that our AMLR factor is distinct from IL-2 comes from the failure of the AMLR supernatants to support an IL-2-dependent cell line, or to function effectively as a co-stimulator in the thymocyte response to Con A or PHA.

As distinct from those studies that detect IL-2 in the AMLR (17, 18), we used different mouse strains, culture conditions, cell populations, and length of culture (3 d instead of 5 d). By duplicating the conditions reported for obtaining IL-2 in the murine AMLR (17, 18), low levels of IL-2 were rarely detectable in AMLR cultures (Dr. M. H. Bocchieri, personal communication). Regardless of whether IL-2 can be obtained in the AMLR under certain conditions, the results of our biological assays demonstrate we are dealing with an active mediator distinct from IL-2.

The production of the AMLR factor by a T cell rules out the possibility that it is IL-1. In addition, the PHA co-stimulator assay has been reported to detect IL-1 (26), and in our hands the Con A thymocyte assay will also detect IL-1. The inactivity of our supernatants in these assays provides further evidence that our factor is not IL-1.

As we cannot directly activate cytotoxic precursors in the thymus, the AMLR

factor may activate mature T helper cells, inducing the production of IL-2, and mediating the generation of cytotoxic activity. Indeed, some IL-2 is detectable in supernatants from assay cultures incubated in the presence of the AMLR factor (J. A. Wolos and B. A. Smith, unpublished observations). Our assay system is composed of only T cells and thymocytes. Therefore, the initial interpretation was that we had bypassed the macrophage (and IL-1) requirement in the generation of CTL. The thymocyte population, however, contains a small percentage of nonlymphoid cells, primarily macrophages and dendritic cells, that have the ability to present antigen (30). The thymic macrophages, when properly stimulated, can produce IL-1 (31). In our studies, when thymic adherent cells were removed by nylon column passage, haptenated thymocytes were still able to induce the generation of substantial cytotoxicity when added into AMLR cultures. However, the AMLR helper factor could no longer facilitate the induction of cytotoxic T cells if the thymocytes had been passed over nylon wool columns before hapten modification. Thus, it appears that a thymic adherent cell is a necessary intermediate in our system.

We therefore propose that in our system, a factor(s) derived from Lyt-1^+23^- T cells, and present in AMLR supernatants, activates thymic macrophages to produce IL-1. In the presence of IL-1 and hapten-modified self, helper T cells are stimulated to synthesize IL-2, which induces the differentiation of cytotoxic effector cells from antigen-sensitized cytotoxic precursors, probably in the presence of immune interferon and other, as yet uncharacterized, factors. This suggested role for the AMLR factor in the cascade of events leading to T cell activation is consistent with current models of cytotoxic cell differentiation.

The characterization of the factor, and its relationship to the different putative IL-3 and to CSF is being examined. Experiments are also underway that will determine the relationships between the T cell producing the AMLR factor, the AMLR-proliferating T cell, and the IL-2-producing T cell.

Summary

In normal mice, the autologous mixed lymphocyte reaction (AMLR) can activate helper T cells that, in the presence of hapten-modified syngeneic cells, can induce a hapten-specific cytotoxic response. Supernatants from AMLR cultures contain a factor(s) that will mediate a cytotoxic T cell response to hapten-altered self. The AMLR factor is effective in facilitating the generation of cytotoxicity only in those cultures containing both T cells and hapten-altered, syngeneic, nonstimulatory cells. Factor production requires an interaction between Lyt-1^+23^- cells and non-T cells (the T cells synthesize it). The AMLR factor does not appear to be interleukin 2 (IL-2) because it does not activate thymocytes in the presence of antigen, nor does it maintain an IL-2-dependent cell line or function in co-stimulator assays. For the AMLR factor to facilitate the generation of cytotoxicity, thymic adherent cells are a necessary intermediate. These data suggest that the factor recoverable from AMLR cultures acts early in the cytotoxic pathway, before IL-1 production.

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