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# T-T-CELL INTERACTIONS DURING IN VITRO CYTOTOXIC ALLOGRAFT RESPONSES

## I. Soluble Products from Activated Ly1$^+$ T Cells Trigger Autonomously Antigen-Primed Ly23$^+$ T Cells to Cell Proliferation and Cytolytic Activity*

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Recent results have indicated that murine cytotoxic T lymphocytes (CTL)[1] reactive against alloantigens as coded for in the K and D region of the major histocompatibility complex (MHC), belong to a minor T-cell subset expressing the Ly23$^+$ phenotype, whereas proliferative responses to I region coded determinants are carried out mainly by T cells expressing the Ly1$^+$ phenotype (1, 2). There is also evidence that in a primary mixed lymphocyte culture (MLC) proliferating Ly1$^+$ cells amplify Ly23$^+$ cell-mediated CTL responses (1), possibly via soluble mediators (3–5).

As to secondary CTL responses, Alter et al. (6) were first to report that CTL specific for H-2K or H-2D alloantigens could be induced by restimulating MLC primed cells with stimulator cells sharing only the I region, but not the K and D region with the stimulating cells used in the primary MLC. Moreover concanavalin A, a mitogen for both Ly1$^+$ and Ly23$^+$ T cells (7) when added to alloantigen primed T cells induces effectively secondary CTL responses with specificity to alloantigens used for primary immunization (8–10). While confirming these observations, we as others (11–13) noted that in vitro the induction of secondary CTL responses could be dissected into two consecutive steps: upon polyclonal (concanavalin A [Con A]) or antigen-specific (secondary MLC) stimulation of murine T cells a soluble product is released into the culture supernate, which upon transfer to alloantigen primed T cells triggers the generation of secondary CTL responses. In analyzing this phenomenon more in detail, it was found that activated Ly1$^+$ T cells release a soluble product which triggers antigen primed Ly23$^+$ T cells to cell proliferation and cytolytic activity.

## Materials and Methods

*Mice.* C57Bl/6 abbreviated C57, B10.A (2R), and B10.A mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. The C57 Bl/6 congenic strain C57Bl/6 Ly-1$^a$/Boy, abbreviated B6/Ly-1.1; and C57Bl/6-Ly2$^a$/Boy abbreviated B6/Ly-2.1, were kindly provided by Dr. E. Boyse (Memorial Sloan Kettering Cancer Center, New York) and further propagated

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[1] *Abbreviations used in this paper:* Ara A, arabinofuranosyladenine; Con A, concanavalin A; CTL, cytotoxic T lymphocytes; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; FITC, fluorescein-isothiocyanate; LPS, lipopolysaccharide; LU, lytic unit; MHC, major histocompatibility complex; MLC, mixed lymphocyte culture; NMS, normal mouse serum; PBS, phosphate-buffered saline; SCIF, secondary cytotoxic T-cell inducing factor.

by strict brother-sister mating in our own mouse facilities. CBA, BALB/c, A.TH, and A.TL mice were obtained from OLAC Ltd., Show's Farm, Blackthorn, England.

*Antisera.* The preparation and use of anti-Thy 1.2 antisera have been described (14). Anti-Ly-1.1 antiserum was prepared according to Shen et al. (15) by immunization of (BALB/c × C57 Bl/6) $F_1$ recipients with (BALB/c × B6/Ly-1.1) $F_1$ thymocytes. Individual sera were tested; only active antisera were pooled, and absorbed with B6 thymocytes. The anti-Ly2.2 serum was a generous gift of Dr. U. Hämmerling, Sloan Kettering Cancer Institute. It was used after adsorption with B6-Ly2.1 thymocytes. To insure the correct specificity of the anti-Ly1.1 and anti-Ly2.2 sera employed in this study, specificity tests were performed with complement-dependent cytotoxicity assays. Out of 146 rabbits tested, serum of 1 selected rabbit served as source of complement. The alloantigenic specificity of anti-Ly1.1 and anti-Ly2.2 sera were tested with B6-Ly1.1 and B6 thymocytes in an eosin dye exclusion complement-dependent cytotoxic test (Fig. 1). Anti-Ly1.1 antiserum was strongly cytotoxic towards B6-Ly1.1 thymocytes, but not against B6 thymocytes, whereas anti-Ly2.2 antiserum was cytotoxic for B6/Ly-2.2 thymocytes, but not for B6/Ly-2.1 thymocytes, demonstrating that both sera were specific for their appropriate alloantigen.

*Use of Anti-Ly-Sera.* Splenic lymphocytes bearing the selectively expressed cell surface alloantigens were suspended in anti-Ly antiserum diluted in phosphate-buffered saline (PBS) supplemented with 5% heat inactivated fetal calf serum (FCS) so that there were $10 \times 10^6$ cells/ml diluted antiserum. Anti-Ly1.1 serum was used at a final dilution of 1:8, and anti-Ly2.2 serum was used at a final dilution of 1:12. The antiserum-treated lymphocytes were incubated at 4°C for 30 min, centrifuged, and resuspended in rabbit complement appropriately diluted in PBS. After incubation at 37°C for 40 min, the surviving cells were resuspended in PBS plus 5% FCS and washed twice.

*MLC.* The MLC was performed with Dulbecco's modified Eagle's medium (DEM) supplemented with 20 mM Hepes, $5 \times 10^{-5}$ M 2-mercaptoethanol, and 5% FCS in 2-ml cultures by using multiculture plates (Linbro FB-24, Tc, Linbro Chemical Company, Hamden, Conn.) as described (16). Spleen cells of the various strains were used as responder and stimulator cells. Before culture, the stimulating cells were X-irradiated with 2,000 rads (Philipps machine RT 200, Fa. Müller, Frankfort, Germany) at a dose rate of 620 rads/min. In primary MLCs, replicates of responder cells ($4 \times 10^6$) were cultured together with stimulator cells ($1 \times 10^6$) in a humified atmosphere of 5% $CO_2$ in air for the desired period of time. Alloantigen primed cells were harvested and viable cells were either tested for cytolytic activity, or redistributed into Linbro culture plates for secondary stimulation. Three different approaches were used for secondary stimulation.

CON A-INDUCED RESTIMULATION. MLC primed responder cells ($3 \times 10^6$) were cultured in the presence of 3 μg Con A/ml (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden).

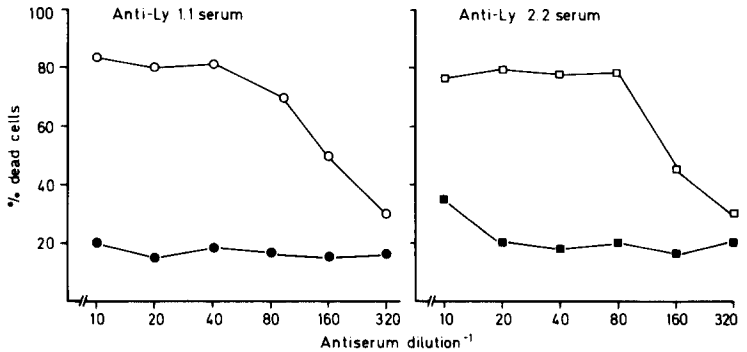


FIG. 1. Complement-dependent dye exclusion cytotoxic test of anti-Ly1.1 and anti-Ly2.2 serum. The anti-Ly1.1 serum was tested on B6/Ly1.1 ●, and B6 ○ thymocytes, the anti-Ly2.2 serum on B6 □ and B6/Ly1.1 ■ thymocytes. $10^6$ cells were incubated in 50 μl diluted antiserum at 4°C for 30 min, thereafter the cells were washed twice and incubated in 50 μl of selected rabbit serum diluted 1:10 at 37°C for 40 min.

STIMULATOR CELL-INDUCED RESTIMULATION. MLC primed responder cells ($3 \times 10^6$) were cultured in the presence of $2 \times 10^6$ X-irradiated (2,000 rads) stimulator cells.

RESTIMULATION BY SECONDARY CYTOTOXIC T-CELL INDUCING FACTOR (SCIF). MLC primed responder cells ($1 \times 10^5$) were cultured in the presence of a given volume of medium supplemented with SCIF. After incubation for 3 days, the lytic activity of viable cells was tested.

*Production of SCIF*

SCIF FROM CON A-STIMULATED LYMPHOCYTE CULTURES. Unless stated differently, splenic responder cells ($10^7$) were cultured in medium without FCS in the presence of 1 μg Con A/ml. After 20 h, the supernate was harvested by decantation, cleared by centrifugation (3,000 *g*, 30 min), and passed over Sephadex G25 to remove Con A. The supernate was used either directly, or after concentration by pressure filtration using Diaflo Ultrafilters with a cut-off for molecules greater than 30,000 daltons (PM 30 filter, Amicon Corp., Scientific Sys. Div., Lexington, Mass.); the supernate was semipurified on Sephadex G100.

SCIF FROM SECONDARY MLC CULTURES. MLC primed responder cells ($3 \times 10^6$) were restimulated with $2 \times 10^6$ stimulator cells in medium containing 1% FCS. After 24 h, the supernate was harvested, cleared from debris by centrifugation (3,000 *g*, 30 min), and concentrated (10-fold) by pressure filtration using Diaflo Ultrafilters with a cut-off for molecules greater than 10,000 daltons (UM 10 filter, Amicon Corp.). SCIF could be stored at −20°C without detectable loss of activity.

*[$^3$H]Thymidine Uptake Assay.* The proliferative response of MLC cells was assayed according to Peck and Bach (17). Lymphocytes were pulse labeled with [$^3$H]thymidine (Amersham Buchler, Frankfurt, Germany, 2 Ci/mM) at 1 μCi/well over 16 h. The cultures were harvested and [$^3$H]thymidine uptake determined as described previously (17).

*Target Cells.* P815 (H-2$^d$) and $EL_4$ (H-2$^b$) tumor cells were grown in suspension cultures. If necessary lipopolysaccharide (LPS) transformed lymphoblasts were used as target cells. Lymphoblasts were obtained by pooling replicate cultures containing $4 \times 10^6$ spleen cells which had been cultured for 72 h in DMEM containing 5 μg/ml LPS (Difco Laboratories, Detroit, Mich.).

*Cytotoxicity Assay.* Various numbers of viable MLC cells or CTL grown in SCIF containing medium were incubated with a constant number ($2 \times 10^4$) [$^{51}$Cr]labeled target cells. Percent-specific $^{51}$Cr-release was calculated according to the formula described (16). For each lymphocyte population tested a dose-response curve was established. By plotting percent lysis obtained versus the log of the ratio of attacker to target cells used, the number of attacker cells could be defined which lysed within 4 h 50% of $2 \times 10^4$ target cells. 1 lytic unit (LU) was arbitrarily defined as the number of lymphoid cells necessary to lyse 50% of the target cells within the assay time.

*Gel Filtration of SCIF on Sephadex G100.* Supernate from Con A-stimulated lymphocytes was first concentrated 50-fold (Diaflo Ultrafilter PM 30). The Sephadex G100 elution characteristic was then estimated using a column of 100 cm length and 1 cm diameter. The Sephadex G100 (Pharmacia, Uppsala, Sweden) was equilibrated with PBS (pH 7.4, conductivity 15 mS) and the column calibrated with dextran blue (MG: $2 \times 10^6$), hemoglobin (MG: 64,000), and cytochrome *c* (MG: 12,500). A 1 ml sample was applied and the column eluted at a constant flow rate of 10 ml/h. Elution was monitored at 280 nm (protein), at 412 nm (hemoglobin), at 625 nm (dextran blue), and at 550 nm (cytochrome *c*). Aliquots of individual fractions were tested for functional SCIF activity. By the addition of trace amounts of [$^3$H]Con A (TRK 497, Amersham-Buchler) to the Con A used, the presence of [$^3$H]Con A in the fractions could also be monitored. Larger batches of semipurified SCIF were eluted from a Sephadex G100 column of 100 cm length and 5 cm diameter.

*Inhibition of DNA-Synthesis by Mitomycin C.* According to previously published work (18), splenic lymphocytes ($20 \times 10^6$/ml) were incubated in DMEM containing 40 μg/ml mitomycin C (Sigma Chemical Co., St. Louis, Mo.) at 37°C for 30 min. Thereafter the cells were washed twice by centrifugation through FCS.

*Inhibition of DNA Synthesis by 9-B-D-Arabinofuranosyladenine (Ara A).* Splenic lymphocytes ($20 \times 10^6$/ml) were incubated in DMEM, containing increasing concentrations of Ara A (H. Mack, Illertissen, Germany), at 37°C for 60 min. Thereafter the cells were washed twice

through FCS. In control experiments, it was demonstrated that Ara A at a final concentration of 5 μg/ml inhibited the DNA-synthesis by more than 90% (19).

*Inhibition of Protein Synthesis by Emetine.* Splenic lymphocytes ($20 \times 10^6$/ml) were incubated in DMEM containing $10^{-4}$ or $10^{-6}$ M emetine (Sigma Chemical Co.) for 30 min at 37°C, to inhibit protein synthesis (20). Protein synthesis was assayed by the incorporation of [$^3$H]leucine (sp act 140 mCi/mM) into acid precipitable lymphocyte proteins. Splenic lymphocytes were incubated in leucine-free Eagle's medium containing graded concentrations of emetine (Sigma Chemical Co.) for 30 min at 37°C. Cells were washed and suspended in leucine-free medium supplemented with nonessential amino acids. $0.5 \times 10^6$ cells were then cultured for 12 h in microtiter plates in 0.2 ml of leucine-free medium containing 1 μCi of [$^3$H]leucine. Acid precipitable [$^3$H]leucine containing proteins were assayed as described (21). While calibrating this system we found that target cells not treated with emetine incorporated [$^3$H]leucine effectively (28,000 ± 3,700 counts/min), the [$^3$H]leucine incorporation into cells treated with $10^{-6}$ M emetine was reduced to 1,900 ± 200 counts/min.

*Detection of Membrane-Bound Alloantigen-Material on MLC-Primed T Lymphocytes.* The method used to detect alloantigen on MLC cells was similar to that first described by Nagy et al. (2). The procedure consisted of three consecutive steps: (*a*) residual B cells in the MLC-primed cells were destroyed by treatment of the cells with rabbit anti-mouse Ig and guinea pig complement in the presence of 0.2% sodium azide; (*b*) purification of viable T cells by centrifugation over Ficoll-Hypaque (density 1.09 g/cm$^3$) at 600 *g* for 15 min; (*c*) detection of receptor-bound alloantigens by direct immunofluorescence. To this, the cells were first incubated with a specific anti-stimulator alloantiserum followed by fluorescein-isothiocyanate (FITC)-labeled rabbit anti-mouse Ig (Behringwerke, Marburg/Lahn, Germany): $0.5 \times 10^6$ MLC-T cells were suspended in ice cold 50 μl of medium supplemented with 5% FCS and 0.2% sodium azide, which contained the appropriate anti-stimulator alloantigen serum at a predetermined optimal dilution, and were incubated for 30 min at 4°C; then the cells were washed three times and were resuspended in 50 μl of medium which contained FITC-labeled rabbit anti-mouse Ig at a predetermined optimal dilution and were incubated at 4°C for 30 min. Thereafter the cells were washed three times and mounted on glass slides and scored under a Zeiss fluorescence microscope (HBO 50 W mercury vapor lamp). Under the conditions used 25–35% of primary MLC T cells bound specifically alloantigen (R. Schawaller, unpublished results).

*Removal of Membrane-Bound Alloantigen by Trypsinization.* Following the procedure of Elliot et al. (22) MLC primed T cells were washed twice in PBS, pH 7.4 without serum, and resuspended in PBS at a concentration of $2 \times 10^7$ cells/ml; to 0.5 ml of the cell suspension an equal volume of PBS containing trypsin (5 mg/ml) (trypsin T 1005, Sigma Chemical Co.) was added, and the mixture was incubated at 37°C for 30 min. To terminate the reaction, 10 ml ice cold DMEM supplemented with 10% FCS was added. After trypsin treatment of primary MLC T cells, the number of alloantigen-positive cells was reduced to 0–5% (R. Schawaller, unpublished results).

## Results

*Induction of Secondary CTL Responses by T-Cell-Derived Mediators.* Apparently, unrelated experimental designs can be used to trigger within alloantigen primed T-cell populations antigen-specific secondary CTL responses. In confirming published work (11–13) we noted that both the T-cell mitogen Con A and I region incompatible stimulator cells were efficient in inducing cytolytic activity within alloantigen primed T cells (Table I). More interestingly, this phenomenon could be dissected into two consecutive steps, that is conditioned medium from Con A activated splenic T cells as well as from secondary MLCs specifically stimulated alloimmune T cells to mount secondary CTL responses (Table I). Because an active supernate was not produced in Con A-stimulated cultures devoid of T cells (Table I), and because active supernates induced secondary CTL responses with specificity for the original alloantigen (Table I), we concluded that upon polyclonal (Con A) or antigen-specific (MLC) stimulation

TABLE I

*Experimental Approaches to Induce Secondary CTL Responses in A.TH Anti-CBA Primed Responder Cells*

| Exp. | Secondary stimulus | Viable cells | Day 3 response | | |
|---|---|---|---|---|---|
| | | | LU/$10^6$ cells (CBA targets) (H-$2^k$) | LU/culture (CBA targets) (H-$2^k$) | LU/culture EL4 targets (H-$2^b$) |
| | | *% input* | | | |
| 1 | CBA | 85 | 101 | 255 | 8 |
| | A.TH | 31 | 2 | 2 | 1 |
| | A.TL | 78 | 76 | 182 | 6 |
| | Con A (3 μg/ml) | 46 | 29 | 14 | 1 |
| 2 | Conditioned medium A (from Con A-stimulated T cells) | 340 | 59 | 22 | 1 |
| | Conditioned medium B (from Con A-stimulated non-T cells) | 32 | 1 | 1 | 1 |
| | Conditioned medium C (from secondary MLCs) | 260 | 54 | 15 | 1 |

Primary A.TH anti-CBA MLC cells were induced in replicate cultures containing 4 × $10^6$ A.TH spleen cells and $10^6$ irradiated (2,000 rads) CBA spleen cells. Exp. 1: after 8 days of culture, 3 × $10^6$ viable cells were restimulated with 2 × $10^6$ irradiated CBA, A.TH, or A.TL spleen cells or Con A (3 μg/ml); on day 11, the cultures were harvested, the viable cell recovery determined, and cytolytic activity assayed against CBA derived LPS induced splenic lymphoblasts and against EL4 target cells. LU were calculated from individual dose-response curves. The data are expressed as LU/$10^6$ cells recovered and as LU/culture. Exp. 2: after 8 days of culture, A.TH anti-CBA MLC cells ($10^5$) were cultured in a 2 ml-vol together with 0.2 ml of conditioned medium A, B, or C for an additional 3 days, thereafter the cytolytic activity of the viable cells was determined. Conditioned medium A was obtained from 20 h-cultures of Con A- (1 μg/ml) stimulated A.TH derived splenic T cells ($10^7$ cells/ml, purified by passage through nylon wool column, 38). Before use the conditioned medium A was passaged through a short (5 × 1.5 cm) Sephadex G25 column to deplete the medium from Con A. Conditioned medium B was obtained similarly as described for A except that no nylon wool-enriched T cells were used, but A.TH spleen cells depleted for T cells by treatment with anti-Thy 1.2 serum plus complement. Conditioned medium C represents a 10-fold concentrate of the supernates of cultures of 3 × $10^6$ MLC primed CBA anti-BALB/c cells (day 7) restimulated for 20 h with 2 × $10^6$ irradiated (2,000 rads) BALB/c spleen cells in DMEM medium supplemented with 1% FCS.

T cells released soluble factors capable of activating alloantigen primed T lymphocytes to become secondary CTL.

*Ly-Phenotype of T-Cell Subsets Involved in T-T Interactions.* The availability of Ly congenic C57Bl/6 mice and of Ly1.1 and anti-Ly2.2-specific antisera allowed us to perform experiments to analyze the T-cell subsets producing the postulated SCIF. The data in Table II indicate that both Ly $1^+$ and Ly $23^+$ T cells are activated by Con A. Upon polyclonal activation SCIF could be detected only in supernates of cultures containing Con A activated Ly $1^+$ T cells. Moreover, removal of Ly$1^+$ T cells from MLC primed responder T cells before antigenic restimulation abolished the capacity of the remaining cells to produce SCIF (Table II). Finally upon restimulation, I-region reactive T responder cells were effective in producing SCIF. Taken together these data strongly suggest that SCIF is derived from activated Ly$1^+$ T cells.

Next the impact of SCIF on purified alloimmune Ly$2.3^+$ T cells was investigated. The data given in Table III indicate that SCIF obtained either from cultures of Con

TABLE II

*Characteristics of T Cells Producing SCIF*

| SCIF producing system | | | | | SCIF induced cytolytic response of CBA anti-BALB/c primed MLC cells (3 day culture) | | |
|---|---|---|---|---|---|---|---|
| Expt. | Origin | Responder cells treatment | Resultant T cells | Stimulation | [$^3$H]thymidine uptake | LU/$10^6$ cells (P815 target) | LU/culture (P815 target) |
| | | | | | *cpm* | | |
| 1 | B6 | NMS | Ly1$^+$ and Ly2$^+$ | — | 1,700 ± 200 | <1 | <1 |
| | B6 | Thy1.2 serum | No T cells | Con A | 900 ± 300 | <1 | <1 |
| | B6 | Ly1.1 serum | Ly1$^+$ and Ly2$^+$ | Con A | 52,000 ± 3,400 | 64 | 14 |
| | B6/Ly1.1 | NMS | Ly1$^+$ and Ly2$^+$ | — | 1,400 ± 400 | <1 | <1 |
| | B6/Ly1.1 | NMS | Ly1$^+$ and Ly2$^+$ | Con A | 45,000 ± 2,900 | 53 | 12 |
| | B6/Ly1.1 | Ly1.1 serum | Ly2$^+$ | Con A | 18,000 ± 2,700 | 3 | <1 |
| | B6/Ly1.1 | Ly2.2 serum | Ly1$^+$ | Con A | 32,000 ± 4,200 | 49 | 11 |
| 2 | Anti-CBA primed B6/Ly 1.1 | NMS | Ly1$^+$ and Ly2$^+$ | CBA cells | ND | 27 | 6 |
| | Anti-CBA primed B6/Ly 1.1 | Ly1.1 serum | Ly2$^+$ | CBA cells | ND | <1 | <1 |
| 3 | Anti-A.TL primed A.TH | — | Ly1$^+$ and Ly2$^+$ | A.TL cells | ND | 47 | 9 |

In Exp. 1, B6 mouse and B6/Ly1.1 mouse derived splenic lymphocytes were first passed over nylon wool column, the effluent cells were treated with the respective antisera plus complement and the surviving cells ($4 \times 10^6$) were cultured in the presence of Con A (3 μg/ml). After 40 h the supernate of the cultures were harvested and viable cells ($0.3 \times 10^6$) were distributed in microtiter plates and pulse labeled with [$^3$H]thymidine for 16 h; the individual supernates harvested were 10-fold concentrated. In Exp. 2, B6/Ly1.1 mouse derived splenic lymphocytes ($4 \times 10^6$) were first primed in a MLC against $10^6$ irradiated (2,000 rads) CBA stimulator cells. After 8 days, the cells were harvested and treated either with normal mouse serum (NMS) plus rabbit complement, or with anti-Ly1.1 serum plus rabbit complement. The surviving cells ($3 \times 10^6$) were recultured with $2 \times 10^6$ CBA stimulator cells. After 20 h, the supernates were harvested and 10-fold concentrated.

In Exp. 3, supernate from I region incompatible A.TH anti-A.TL MLCs was harvested 20 h after secondary restimulation and 10-fold concentrated.

In a second step, the concentrated supernates of all three experiments (0.2 ml) were added to subcultures of 8 day MLC primed CBA anti-BALB/c cells ($1 \times 10^5$ per culture). After 3 days the lytic activity of the recovered viable cells was tested against [$^{51}$Cr]labeled P815 targets. LU were calculated from individual dose-response curves obtained.

A activated T cells or from I-region incompatible MLCs 24 h after secondary restimulation triggered antigen primed Ly2.3$^+$ cells to differentiate into CTL. These results imply that polyclonally as well as antigen activated Ly1$^+$ cells produce SCIF which upon transfer to alloantigen primed Ly2.3$^+$ T cells cause secondary CTL responses to be generated.

*Optimal Conditions for the Production of the Ly1$^+$ T-Cell-Derived SCIF.* Of the results obtained studying optimal conditions for the production of SCIF, three informative experiments are depicted in Fig. 2 using polyclonally (Con A) activated CBA splenic lymphocyte cultures. Accordingly SCIF activity was highest in the culture supernates when $10 \times 10^6$ splenic cells were cultured with 1 μg Con A for 24 h in the absence of FCS.

Because SCIF activity appeared in the supernates of Con A activated T cells within 12–24 h (Fig. 2), the metabolic requirements for the production of SCIF were studied. Before Con A stimulation splenic lymphocytes were treated either with mitomycin C (to block DNA synthesis [18]), 9-B-D-Ara A to block DNA synthesis (19), and emetine (to block protein synthesis [20]) at concentrations previously calibrated for selective inhibition of the metabolic pathway in question (Materials and Methods). Whereas mitomycin C and Ara A treatment did not affect the capacity to produce SCIF, emetine pretreated cells were unable to do so (Table IV). It is surprising that X-irradiation did not affect the capacity of T cells to produce SCIF (Table IV).

TABLE III

*Effect of SCIF on Alloantigen Primed Ly2$^+$ T Cells*

| SCIF from | Day 3 response | |
|---|---|---|
| | LU/$10^6$ cells | LU/culture |
| None | 2 | 1 |
| Con A activated CBA spleen cells | 137 | 46 |
| Secondary anti-ATL primed ATH cells | 98 | 35 |

In a first step, B6/Ly1.1 responder cells were primed in a MLC towards BALB/c stimulator cells. After 8 days, cells were harvested, treated with anti-Ly1.1 serum plus complement, the remaining cells were distributed into subcultures ($1 \times 10^5$/culture) and 0.2 ml of 10-fold concentrated supernates from polyclonally activated (Con A) or antigen-specific (MLC) activated cells were added. After 3 days, the lytic activity of viable cells recovered was tested against [$^{51}$Cr]labeled P815 targets. LU were calculated from individual dose-response curves.

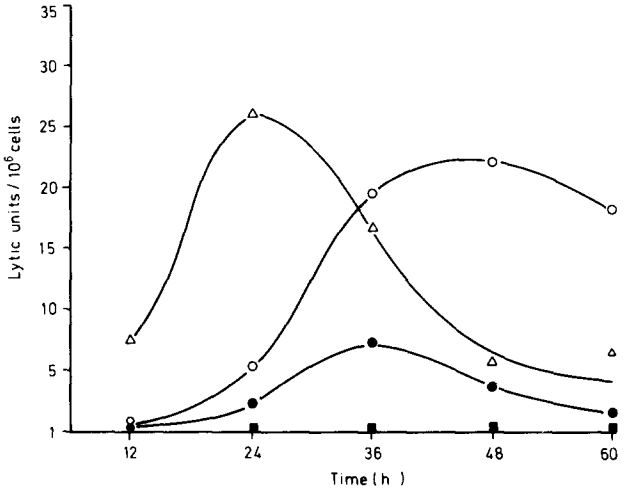


FIG. 2. Effect of cell/volume ratio and Con A concentration on the production of SCIF. In a first step, CBA splenic lymphocytes were cultured in the absence (■) or presence of Con A for various lengths of time under several conditions. At the time intervals given, the supernate of individual cultures was harvested. (Δ), $10 \times 10^6$ cells per culture, without FCS, 1 μg ConA per ml, (○) $4 \times 10^6$ cells per culture, 2% FCS, 5 μg Con A per 2 ml, (●) $2 \times 10^6$ cells per culture, 2% FCS, 5 μg Con A per 2 ml. In a second step, MLC primed CBA anti-BALB/c cells (day 8 cells) were distributed into Linbro culture plate cells ($1 \times 10^5$ viable cells) and cultured with the individual supernates at a 50% (vol/vol) final concentration. At the end of a 3-day culture period, lytic activity of the CBA anti-BALB/c cells was tested against [$^{51}$Cr]labeled P815 target cells. LU per culture were calculated from individual dose-response curves.

*Functional Activity of SCIF.* A batch of semipurified SCIF was prepared by first culturing replicates of CBA mouse derived splenic cells ($10 \times 10^6$) together with Con A (1 μg/ml) and trace amounts of [$^3$H]labeled Con A in serum-free medium for 24 h. The culture supernate was concentrated 50-fold using an Amicon Filter with a cut-off for molecules above 30,000 daltons. Upon filtration on Sephadex G100 groups of individual fractions were tested for SCIF activity, the positive fractions were selected, pooled, 50-fold concentrated, and this batch of SCIF was used in the following experiments. The data given in Fig. 3 demonstrate the elution pattern of SCIF on Sephadex G100 calibrated with dextran blue, hemoglobin, and cytochrome C.

TABLE IV

*Metabolic Requirements for the Production of SCIF*

| Treatment of SCIF producing cells | Day 3 response of CBA anti-BALB/c primed cells | |
|---|---|---|
| | LU/$10^6$ cells | LU/culture |
| None | 54 | 17 |
| Mitomycin C (40 μg/ml) | 49 | 12 |
| Ara A (120 μg/ml) | 52 | 11 |
| Ara A (60 μg/ml) | 47 | 13 |
| Emetine ($10^{-4}$M) | <1 | <1 |
| Emetine ($10^{-6}$M) | 3 | <1 |
| X-irradiation (1,000 rads) | 36 | 10 |
| X-irradiation (3,000 rads) | 24 | 5 |

CBA mouse derived splenic lymphocytes were either X-irradiated or pretreated with the metabolic inhibitors listed. After washing the cells ($10^7$/well) were cultured in FCS-free medium containing 1 μg ConA/ml. After 24 h the supernates were harvested. In a second step, MLC primed CBA anti-BALB/c cells (day 8 cells) were distributed into culture wells (1 × $10^5$ viable cells) and cultured for 3 days with the individual supernates at a 50% (vol/vol) final concentration. Thereafter lytic activity of the subcultures was tested against [$^{51}$Cr]labeled P815 target cells. LU were calculated from individual dose-response curves.

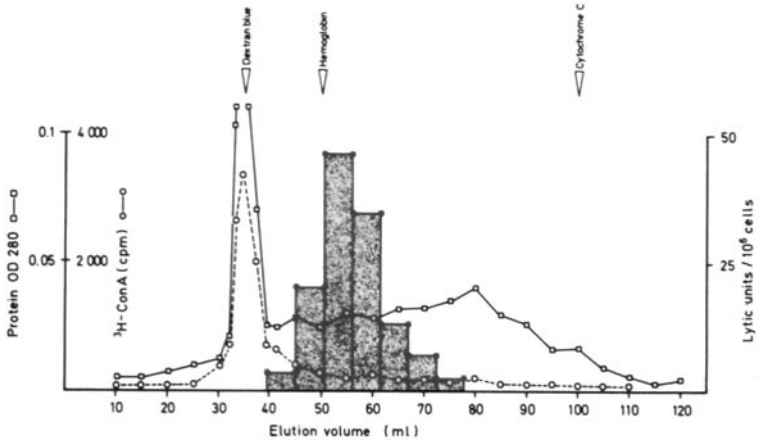


FIG. 3. Elution pattern of SCIF on Sephadex G100 (for details see Materials and Methods).

Accordingly the fractions containing SCIF activity eluted in a position to the right of that of hemoglobin (MG: 64,000). It should be stressed that semipurified SCIF did not contain detectable amounts of [$^3$H]Con A.

SELECTIVE MITOGENICITY OF SCIF FOR ALLOANTIGEN PRIMED T LYMPHOCYTES. To test whether semipurified SCIF acts as a mitogen on its own, graded concentrations of SCIF were added to either normal or CBA anti-BALB/c primed MLC cells. Cell proliferation ([$^3$H]thymidine uptake) and induction of cytotoxic activity (towards [$^{51}$Cr]labeled P 815 targets) were assayed 3 days later. As can be seen in Table V, semipurified SCIF induced in CBA anti-BALB/c primed MLC cells strong proliferative and cytotoxic responses. Moreover, there was a clear dose-dependent relationship

TABLE V

*Selective Mitogenicity of SCIF for Alloantigen Primed T Cells*

| Semipurified SCIF added | Day 3 response of unprimed T cells | | Day 3 response of CBA anti-BALB/c primed T cells | |
|---|---|---|---|---|
| | [$^3$H]thymidine uptake | LU/culture | [$^3$H]thymidine uptake | LU/culture |
| *% final concentration* | *cpm* | | *cpm* | |
| None | 3,100 ± 300 | <1 | 1,200 ± 400 | <1 |
| 100 | 5,300 ± 1,900 | <1 | 86,000 ± 9,400 | 47 |
| 50 | 4,700 ± 200 | <1 | 97,000 ± 2,400 | 31 |
| 10 | 7,100 ± 1,300 | <1 | 43,000 ± 200 | 13 |
| 2 | 3,600 ± 900 | <1 | 26,000 ± 1,400 | 2 |

Normal CBA spleen cells ($3 \times 10^6$) or anti-BALB/c MLC primed (day 8) CBA spleen cells ($1 \times 10^5$) were distributed in Linbro culture plate wells. Semipurified SCIF was added in the concentrations given. After 3 days the cells of the various groups were harvested, their viability was determined, and part of the cells were used to assay for lytic activity towards [$^{51}$Cr]labeled P815 cells. LU were calculated from individual dose-response curves. The remaining cells of the individual groups were suspended in that a volume of culture medium, which resulted in the group containing 100% SCIF in $2 \times 10^5$ alloantigen primed cells/0.2 ml. 0.2 ml replicates of the individual groups were cultured in microtiter plates and pulse labeled with [$^3$H]thymidine (relative comparison on a culture basis).

between the magnitude of the proliferative and cytotoxic responses induced and the amount of SCIF added. In contrast, SCIF was found not to be significantly mitogenic for unprimed lymphocytes, nor did it induce cytolytic activity.

LONG-TERM GROWTH OF LY 2.3-POSITIVE CTL. Repeated addition of semipurified SCIF to C57Bl/6 anti-BALB/c immune MLC cells devoid of Ly1$^+$ T lymphocytes supported continuous growth of the remaining Ly2.3$^+$ T cells. More interestingly the proliferating cells exhibited high cytotoxic activity over a time period of up to 30 days (Fig. 4). It is our experience that such a regimen resulted during the first 2 wk in a dramatic increase in the frequency of viable cytotoxic Ly23$^+$ T cells. The reason, why after 30–40 days the cells become refractory to the effect of SCIF, it not clear. In one experiment we were able to propagate MLC primed responder cells with high cytolytic activity for up to 7 wk (data not given).

SPECIFICITY OF SCIF GROWN CTL. To test for the antigen specificity of SCIF grown CTL, the lytic activity of MLC primed CTL (day 5 cells) was compared to that of CTL grown and expanded in medium containing semipurified SCIF. The results obtained (Table VI) indicate that the antigen specificity of SCIF induced CTL was comparable to that of MLC primed CTL.

FUNCTIONAL ACTIVITY OF SCIF IS NOT H-2 RESTRICTED. The results given in Table VII indicate that SCIF obtained from Con A activated splenic lymphocytes of different H-2 haplotypes stimulated almost equally well CTL generation of CBA anti-BALB/c primed MLC-cells. Similarly supernates of secondary MLC involving cells of different H-2 haplotypes were effective. On the other hand, SCIF obtained from either Con A activated or I region activated A. TH mouse responder cells also stimulated induction of secondary CTL responses of H-2 different alloimmune T-cell populations. The results given in Table VII also contain an example of experiments demonstrating that alloimmune T cells, which were centrifuged on Ficoll-Hypaque

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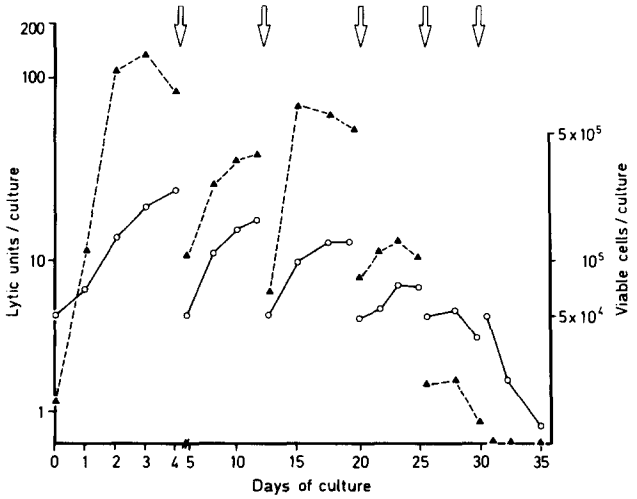


FIG. 4. MLC primed B6/Ly1.1 anti-BALB/c cells (day 8) were treated with anti-Ly1.1 antiserum plus complement to delete for Ly1.1$^+$ cells. The remaining alloantigen primed Ly2.3$^+$ T cells were distributed into Linbro culture plate wells (5 × 10$^4$ per culture) and seimpurified SCIF (5% vol/vol) was added. Viability and lytic activity of the cells was scored at the time intervals given. The arrows indicate the time points, when the cultures were split and subcultured (5 × 10$^4$ cells/culture) in fresh medium, containing 5% (vol/vol) SCIF. (▲) LU/culture (○) viable cells.

TABLE VI

*Specificity of SCIF Grown B10.D2 Anti-B10.A (2R) CTL*

| Effector cells | Cytolytic activity (LU/10$^6$ cells) towards | | | | | |
|---|---|---|---|---|---|---|
| | BIO.A (2R) | BALB/c | A.TH | C57BL/6 | CBA | A.SW |
| MLC primed CTL | 39 | <1 | 2 | 26 | 18 | <1 |
| SCIF grown CTL | 43 | <1 | 4 | 35 | 23 | <1 |

B10.D2 anti-B10.A (2R) MLC primed cells (day 8) were grown in SCIF containing medium and were subcultured at day 12 and 16. Lytic activity of SCIF grown CTL was tested (day 17) against a panel of H-2 different, LPS induced splenic target cells. In addition, the lytic activity of MLC primed B10.D2 anti-B10.A (2R) cells (day 5 cells) was tested against the same target cells. LU were calculated from individual dose-response curves.

(to remove dead cells originating from the allogenic stimulator cells) followed by trypsinization (to remove receptor bound alloantigens) nevertheless developed lytic activity upon addition of SCIF. The results, therefore, suggest that the functional activity of SCIF is neither antigen specific nor H-2 restricted. Moreover, SCIF induced secondary CTL responses in an apparently autonomous fashion, that is in the absence of detectable alloantigens.

SCIF-SUSCEPTIBILITY OF ALLOIMMUNE T CELLS AT VARIOUS STATES OF DIFFERENTIATION. CTL induced in primary MLCs are blast lymphocytes which revert with time to medium sized nonlytic secondary CTL (23–25). It was therefore of interest to test the influence of semipurified SCIF upon alloimmune T cells at various time points after primary sensitization. As can be seen in Table VIII, addition of semipurified SCIF to cultures containing 8 day primed MLC cells resulted in a dramatic increase of lytic activity. More interestingly semipurified SCIF also induced cytolytic activity in nonlytic (day 15) secondary MLC cells.

TABLE VII

*Functional Activity of SCIF*

| Supernates of Con A activated cells* | Day 3 response of H-2$^k$ anti-H-2$^d$/primed cells LU/culture (P815 targets) | Supernates from secondary MLC‡ | Day 3 response of H-2$^k$ anti-H-2$^d$/primed cells LU/culture (P815 targets) |
|---|---|---|---|
| CBA | 26§ | CBA Anti-BALB/c | 24 |
| CBA (T-cell depleted) | 1 | | |
| C57 | 31§ | CBA Anti-C57 | 14 |
| C57 (T-cell depleted) | 1 | C57 Anti-BALB/c | 12 |
| BALB/c | 45 | BALB/c Anti-C57 | 17 |
| BALB/c (T-cell depleted) | 1 | | |
| B10 A | 29 | None | 1 |
| B10 A (T-cell depleted) | 1 | | |

* Splenic lymphocytes ($10^6$ cells/culture) treated with either mouse serum plus complement or anti-Thy 1.2 serum plus complement were cultured in the presence of 1 μg Con A/ml without FCS. Supernates were harvested 24 h later, 10 times concentrated, and filtered on Sephadex G25.

‡ MLC primed responder cells ($2 \times 10^6$ cells) were restimulated with stimulator cells ($2 \times 10^6$ cells) used for primary sensitization. Supernates were harvested 24 h later and five times concentrated. To test for SCIF activity MLC primed H-2$^k$ anti-H-2$^d$ cells (day 8) were distributed into Linbro wells ($1 \times 10^5$ viable cells) and cultured in the presence of 10% (vol/vol) of SCIF for 3 days. Thereafter cytolytic activity was determined towards [$^{51}$Cr]labeled P815 target cells.

§ Refers to MLC primed CBA anti-H-2$^d$ responder cells devoid of detectable alloantigens. Removal of receptor bound alloantigens was achieved by trypsinization of the cells. MLC primed cells were first purified from dead cells by centrifugation on Ficoll-Hypaque. Whereas 20–25% of viable cells carried H-2$^d$ alloantigens as detected by the indirect immunofluorescence technique, after trypsinization of the cells the number of alloantigen-positive cells was reduced to 0–2% (for details see Materials and Methods).

TABLE VIII

*Influence of SCIF Upon Nonlytic Secondary H-2$^k$ anti-H-2$^d$ CTL*

| Days after primary sensitization | None | Cytolytic response (LU/culture) Addition of SCIF at day | | | | | |
|---|---|---|---|---|---|---|---|
| | | 8 | 11 | 12 | 13 | 14 | 15 |
| 5 | 107 | | | | | | |
| 7 | 46 | | | | | | |
| 8 | 9 | | | | | | |
| 9 | 3 | 24 | | | | | |
| 10 | <1 | 76 | | | | | |
| 11 | <1 | 120 | | | | | |
| 12 | <1 | 36 | 4 | | | | |
| 13 | — | 4 | 39 | 2 | | | |
| 14 | — | <1 | 117 | 14 | 5 | | |
| 15 | <1 | — | — | 87 | 19 | 3 | |
| 16 | — | <1 | 54 | 76 | 31 | 28 | — |
| 17 | — | — | 2 | 32 | 47 | 72 | 13 |
| 18 | — | — | 5 | 7 | 19 | 39 | 22 |
| 19 | — | — | <1 | <1 | <1 | 2 | 11 |
| 22 | — | — | <1 | — | <1 | <1 | 4 |
| 25 | — | — | — | — | — | <1 | <1 |

CBA mouse derived splenic lymphocytes ($4 \times 10^6$) were stimulated towards X-irradiated BALB/c stimulator cells ($1 \times 10^6$) in a primary MLC. At various time points after stimulation, 0.1 ml semipurified SCIF was added to individual groups. At the time points indicated lytic activity was tested against [$^{51}$Cr]labeled P815 cells and LU were calculated from individual dose-response curves.

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## Discussion

The new aspect emerging from this study is the finding that in vitro activated Ly1$^+$ T cells release a product which in turn is capable of triggering alloantigen primed Ly2.3$^+$ T cells to cell proliferation and cytolytic activity. While both polyclonal (Con A) and antigen-specific (MLC) stimulation of Ly1$^+$ T cells resulted in the production of the SCIF, once induced the functional activity of SCIF was not antigen specific nor H-2 restricted. Semipurified SCIF acted as mitogen for alloantigen primed, but not for unprimed CTL precursor cells. SCIF allowed exponential growth and long-term propagation of cytolytic Ly2.3$^+$ T cells with specificity of the alloantigens used for primary sensitization. Because SCIF induced activation of alloantigen primed Ly2.3$^+$ T cells took place in the absence of detectable alloantigens, the results describe a pathway whereby nonspecific factor(s) derived from activated Ly1$^+$ T cells induced Ly2.3$^+$ T-cell-mediated, antigen-specific, secondary CTL responses in an apparently autonomous manner.

It has been reported that conditioned medium, derived from polyclonally (phytohemagglutinin, Con A) activated lymphocytes, supports long-term proliferation of CTL presensitized against alloantigens or tumor-specific transplantation antigens (11, 12). These results extended previous findings, according to which Con A induces secondary CTL responses when added to alloimmune T cells (9, 10). A possible corollary represents the observation that supernates from secondary MLCs allowed the induction of antigen-specific secondary CTL responses when transferred to nonlytic MLC primed T cells (13, 26). While confirming these results (Tables I and II) we noted that supernates from I-region incompatible MLCs were equally effective (Table II). Because I-region reactive Ly1$^+$ T cells are preferentially mediating the proliferative response observed in H-2 incompatible MLCs, we supposed that polyclonally (Con A) as well as antigen specifically (MLC) activated Ly1$^+$ T cells may release mediators capable of inducing secondary CTL responses in alloimmune T cells. The results presented in Table II are fully consistent with this view. While the data imply that polyclonal or antigen-specific activation of Ly1$^+$ T cells, but not of Ly2.3$^+$ T cells results in the production of SCIF, no information is available in regard to the Ly123$^+$ T-cell subset. Because Ly123$^+$ T cells proliferate in MLCs, which contain lymphocytes from H-2 mutant mice (27), their capacity to produce SCIF has yet to be determined.

When polyclonally induced SCIF was filtered through Sephadex G100, it eluted under the experimental conditions used in a position in between that of hemoglobin (MG: 64,000) and cytochrome *c* (MG: 12,500) (Fig. 2). This batch of semipurified SCIF did not contain detectable amounts of Con A (Fig. 3), nor was it mitogenic when cultured together with normal T cells (Table V). SCIF was produced by Con A activated T cells within 24 h (Fig. 2). SCIF production was dependent on an intact protein metabolism but DNA metabolism was not required. Even X-irradiated T cells were capable of producing SCIF (Table IV). Assuming SCIF turns out to be identical with the helper factor produced by the Ly1$^+$ T cell during primary CTL responses (3–5), this latter finding may explain why purified Ly2.3$^+$ responder cells are capable of mounting primary CTL responses, provided the X-irradiated stimulator cells used contain Ly1$^+$ cells (28, our unpublished results). Under such experimental conditions help may be provided by the Ly1$^+$ T stimulator cells (internal back-stimulation) and therefore may bypass the requirement for Ly1$^+$ T helper cells.

The functional activity of polyclonally (Con A) as well as of antigen-specific (MLC) induced SCIF was found to neither antigen-specific nor H-2 restricted (Table V). No molecular information is yet available in regard to their possible molecular identity, but both factors are $Ly^+$ T-cell derived and trigger alloantigen primed T cells (Table II). Similar findings have recently been obtained when human or murine MLC supernates were transfered to MLC primed lymphocytes (13, 29).

Semipurified SCIF acted as mitogen for alloantigen primed $Ly2.3^+$ T cells at various time points after primary sensitization (Table VIII), but not for unprimed T cells (Table V). Unlike polyclonal Con A stimulation which is known to reverse with time (30), SCIF added at 3-day intervals induced exponential growth of alloantigen primed $Ly2.3^+$ T cells over a 3–4 wk period. Consequently, to avoid a crowding effect the cells had to be subcultured each 4–6th day. The interesting point, however, is that the proliferating cells exhibited high cytolytic activity (Fig. 4). Although the magnitude of cytolytic activity suggested that the majority of the proliferating T cells might exhibit cytolytic activity, the actual frequency of CTL has yet to be determined. Furthermore, it is not known whether the clonal composition of SCIF-induced CTL is at variance with MLC primed CTL. However, SCIF-grown CTL exhibited the same specificity for these alloantigens used for primary sensitization as did MLC primed CTL (Table VI).

To explain primary CTL responses, we (31), Bach et al. (32), and Cantor and Simpson (33), have proposed a two signal model. Accordingly signal 1 is provided by binding of alloantigen to clonally distributed receptors on $Ly2.3^+$ T cells, whereas signal 2 is nonspecific and can be delivered by I-region reactive $Ly1^+$ T cells. Indeed there is increasing evidence for MLC derived nonspecific factors being able to amplify primary CTL responses (3–5). Unlike the primary CTL responses we suggested, the secondary CTL responses are preferentially induced by alloantigens (signal 1) (25, 34). On the basis of the results presented here this view has now to be modified for the following reasons: it is unlikely that after repeated subcultivation of SCIF-grown CTL, receptor-bound alloantigen originating from the primary MLC is still present on the proliferating CTL, thereby providing signal 1. Moreover using the same technique as Nagy et al. (2), we were unable to detect alloantigen on CTL grown in medium containing SCIF (Table VI). Because trypsin treatment of primary MLC-derived CTL removes receptor-bound alloantigen (22) and because trypsin-treated alloimmune T cells responded to SCIF as did untreated cells (Table VI), we are left with the conclusion that in the absence of alloantigen (signal 1) the nonspecific signal 2 (SCIF) provided by $Ly1^+$ T cells can trigger cell proliferation and secondary CTL responses within alloimmune $LY2.3^+$ T cells in an apparently autonomous fashion.

The present report describes a pathway during which $Ly2.3^+$ T-cell-mediated secondary CTL responses are induced via a soluble product (SCIF) derived from activated $Ly1^+$ T cells. In our view SCIF is likely to be identical with the blastogenic factor described by Uotila et al. (29) and the costimulator factor analyzed by Paetkau et al. and Shaw et al., (35, 36) and Lafferty and Woolnough (37). It is tempting to speculate that the $Ly1^+$ T-cell-derived SCIF as defined here is identical to the amplifying factor acting as signal 2 in primary CTL responses (3–5). Indeed results of recent studies conducted in our laboratory have suggested that SCIF facilitates and amplifies the generation of CTL in a primary MLC using purified $Ly2.3^+$ T cells as responder cells and UV-light inactivated allogeneic lymphocytes as stimulator cells.

While work is in progress to define the biological properties of SCIF in primary CTL responses, the availability of the Ly1$^+$ T-cell-derived SCIF has practical implications. Because of its nonspecific activity SCIF provides means to grow in mass cultures CTL of a given specificity. Such CTL may be useful in vivo as effector cells, or as antigen to induce antiidiotypic immune responses. SCIF may also be used to grow in semisolid agar individual colonies of CTL as a first step to establish CTL of defined clonal origin.

## Summary

Secondary murine cytotoxic T lymphocyte responses from alloantigen-primed T cells can be induced in vitro by apparently unrelated regimens, such as addition of either concanavalin A (Con A), conditioned medium from Con A stimulated lymphocyte cultures, conditioned medium from secondary mixed lymphocyte cultures (MLC), or stimulator cells sharing only the I-region with the stimulating cells used for primary sensitization. We now report that upon polyclonal (Con A), or antigen-specific (MLC) stimulation, Ly1$^+$ T cells release a factor, which in turn triggers alloantigen primed Ly23$^+$ T cells to proliferation and cytolytic activity. The secondary cytotoxic T lymphocyte inducing factor (SCIF) is produced within 24 h. For its production, an intact protein metabolism, not DNA metabolism, is required. Once induced, the functional activity of SCIF is nonspecific and not H-2 restricted. SCIF allows exponential growth and long-term propagation of cytolytic Ly23$^+$ T cells with specificity to alloantigens used for primary sensitization. SCIF induced activation of alloantigen primed Ly23$^+$ T cells does not require the presence of alloantigens. The results therefore reveal a process by which Ly1$^+$ T-cell-derived nonspecific factor(s) induce autonomously Ly23$^+$ T-cell-mediated, antigen-specific, cytotoxic T lymphocyte responses.

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