THE IN VITRO GENERATION AND SUSTAINED CULTURE OF NUDE MOUSE CYTOLYTIC T-LYMPHOCYTES*

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We recently reported the development of culture methodologies that allow for the sustained in vitro exponential growth of both murine and human, antigen-specific cytolytic T-lymphocyte lines (CTLL)[1] (1, 2). Long-term culture of CTLL was thoroughly dependent on the continual presence of a T-cell growth factor (TCGF) produced by T-cell mitogen or antigen-stimulated, normal murine, rat or human mononuclear cells. The ability of TCGF to allow for the indefinite culture of differentiated effector T cells prompted an investigation regarding several of the biological characteristics of TCGF.

We found that TCGF production was T-cell specific in that only T-cell mitogenic or antigenic stimulation resulted in TCGF release by mononuclear cells (3). In addition, TCGF production required the presence of both mature T cells and adherent cells. Removal of Thy-1 antigen-positive splenic T cells (3) or adherent splenic cells markedly decreased TCGF production.[2] The proliferative response to TCGF was also found to be T-cell specific. Only cells previously activated by T-cell mitogens or antigens were found to absorb TCGF activity and to proliferate in an indefinite exponential fashion (4). These findings suggested that TCGF served as the second signal in the T-cell immune response and functioned to mediate the proliferative expansion of antigen- or mitogen-activated T-cell clones.

Of particular interest were the observations that murine thymocytes produced relatively little TCGF and exhibited weak proliferative responses after stimulation with concanavalin A (Con A), whereas cortisol-resistant thymocytes produced TCGF and mediated proliferative responses identical to those generated by normal spleen cells.[2] We also observed that, although thymocytes produced little TCGF, they were capable of mounting a Con A-induced proliferative response equal in magnitude to that produced by Con A-stimulated spleen cells, provided TCGF was supplied exogenously.[2] These observations suggested that the limiting factor behind poor Con

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[1] *Abbreviations used in this paper:* CTLL, cytotoxic T-lymphocyte lines; Con A, concanavalin A; DEAE, diethylaminoethyl; FCS, fetal calf serum; HBSS, Hanks' buffered salt solution; HP-1, helper peak-1; ^{3}H-Tdr, tritiated thymidine; LC, lytic capacity; LE, lytic efficiency; LMC, lymphocyte-mediated cytolysis; LU, lytic unit; MLC, mixed lymphocyte culture; pp, partially purified; SRBC, sheep erythrocytes; TCGF, T-cell growth factor.

[2] Smith, K. A., S. Gillis, F. W. Ruscetti, P. E. Baker, and D. McKenzie. The production and action of T-cell growth factor. Manuscript submitted for publication.

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A-induced thymocyte proliferation was a relative lack of those cells capable of TCGF production.

If this supposition were correct, then one might expect that immature thymic precursors, such as those present in the athymic nude mouse, might also respond to immunologic stimuli provided TCGF were present. In this communication, we present the results of experimentation which show that nude mouse spleen, lymph node, and bone marrow cells are capable of T-cell mitogen-induced proliferative responses, provided mitogen sensitization is performed in the presence of TCGF. Furthermore, nude mouse spleen cells, when stimulated in TCGF-supplemented mixed lymphocyte culture (MLC), give rise to Thy-1 antigen-positive effector cell populations capable of mediating significant in vitro cytolysis of alloantigen-specific target cells. Nude mouse cytolytic lymphocytes have been maintained in TCGF-dependent culture for over 3 mo during which time they have continued to demonstrate antigen-specific cytolytic reactivity. The observation that nude mouse pre-T-cell populations can respond to in vitro alloantigen sensitization lends supportive evidence that: (*a*) antigen-reactive pre-T-cells are present in the spleens of athymic mice; and, (*b*) a fundamental reason behind the T-cell immunodeficiency of the nude mouse is its inability to produce TCGF.

Materials and Methods

Animals. BALB/c female, athymic, nu/nu (nude) mice, 5–7 wk of age were purchased from ARS Sprague-Dawley, Solon, Ohio. NIH female, nude mice, 5–7 wk of age were purchased from Harlan Industries, Indianapolis, Ind. Normal BALB/c, C57Bl/6, DBA/2, DBA/1, Ajax, and SJL female mice, 4–8 wk of age were purchased from the Jackson Laboratory, Bar Harbor, Maine. Charles River CD rats, 6–10 wk of age were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass.

TCGF Production. TCGF for use in the routine maintenance of both normal mouse and nude mouse CTLL was produced by the 48-h stimulation of CD rat spleen cells (1×10^6 cells/ml) with Con A (5 μg/ml, Miles-Yeda Laboratories, Rehoveth, Israel) as previously described (3). In experimentation designed to monitor TCGF produced by normal murine spleen, as well as by nude mouse spleen, bone marrow, and lymph node cells, responding cell populations (1×10^7 cells/ml) were stimulated with Con A (2.5 μg/ml) for various time periods (Results). Cells were cultured at 37°C in RPMI 1640 medium, supplemented with 10% heat-inactivated (56°C for 30 min) fetal calf serum, (FCS, Grand Island Biological Co., Grand Island, N. Y.) 2.5×10^{-5} μM/ml 2-mercaptoethanol, 300 μg/ml fresh L-glutamine (Grand Island Biological Co.), 50 U/ml penicillin-G, and 50 μg/ml gentamicin, in a humidified atmosphere of 5% CO_2 in air. At the conclusion of the culture periods, the cells were removed by centrifugation (1,000 *g* for 10 min) and the supernates assayed for TCGF activity.

Preparation of Partially Purified (pp) TCGF. Conditioned medium containing TCGF activity was partially purified to remove Con A. Rat TCGF was precipitated by the addition of solid ammonium sulfate to a 70% concentration. The resulting precipitate was applied to a diethyl-aminoethyl (DEAE) cellulose column at pH 8.2. TCGF activity was found to elute from the column between 15 and 35 mM phosphate salt concentration. These fractions contained less than 1% of the starting protein and radioiodinated Con A was not found to elute in these fractions.[3]

TCGF Microassay. TCGF activity was assayed as previously described (3) using either CTLL 1 or CTLL 2 cells as the indicator cell population (1). The results were quantified by probit analysis (3) and expressed as units of activity based on a standard rat TCGF preparation.

Tritiated Thymidine (^{3}H-Tdr) Incorporation Assays. Murine cells (100 μl, 1×10^6 cells/ml) were seeded in triplicate into 96-well microtiter plates (No. 3596, Costar, Data Packaging, Cam-

[3] McKenzie, D., S. Gillis, W. Culp, and K. A. Smith. Manuscript in preparation.

bridge, Mass.) in Click's medium (Altick Associates, Hudson, Wis.) supplemented with normal mouse serum, 25 μM/ml Hepes buffer (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), 16 μM/ml $NaHCO_3$, 300 μg/ml fresh L-glutamine, 50 U/ml penicillin-G, and 50 μg/ml gentamicin. Cell populations included NIH and BALB/c nude mouse spleen, bone marrow, and lymph node cells. The following stimulants were added in 100-μl vol: (*a*) supplemented Click's medium, (*b*) Con A (5 μg/ml), (*c*) pp-TCGF, and (*d*) pp-TCGF plus Con A (5 μg/ml). Microplates were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. On day 4 of culture, 0.5 μCi of ^{3}H-Tdr (Schwartz/Mann Div., Becton, Dickinson, & Co., Orangeburg, N. Y., sp act, 1.9 Ci/mM) was added to each well and the incubation continued for 4 h. Cultures were harvested onto glass fiber filter strips and ^{3}H-Tdr incorporation determined as previously described (3). Results are expressed as the mean counts per minute ± 1 SD of triplicate cultures.

CTLL Culture. Both normal and nude mouse CTLL were seeded at 1×10^4 cells/ml in 50% TCGF/50% supplemented Click's medium (vol/vol) in either 25 cm^2 or 75 cm^2 plastic tissue culture flasks (Nos. 3013, 3024, Falcon Labware Div. of Becton-Dickinson, Inc., Oxnard, Calif.). After 3 or 4 d of culture, when the cells had reached a density of $1–2 \times 10^5$ cells/ml, the cultures were subcultured in fresh Click's medium/TCGF (50%/50%, vol/vol) to 1×10^4 cells/ml.

Nude Mouse MLC Stimulation. MLC were conducted as previously described (5). Briefly, 10 ml of either NIH or BALB/c nude mouse spleen cells (2.5×10^6 cells/ml) were mixed with an equal volume and concentration of x-irradiated (1,500-rad-cobalt source) C57Bl/6 spleen cells. MLC were conducted in 2% FCS-supplemented Click's medium and cultured upright in 30-cm^2 tissue culture flasks (No. 3012 Falcon Labware Div. of Becton-Dickinson, Inc.) in a humidified atmosphere of 5% CO_2 in air at 37°C. On successive days of culture (days 0–4), 10 ml of tissue culture medium was removed from one of five replicate flasks and replaced with 10 ml of TCGF. After 5 d of MLC stimulation, viable effector cells from both TCGF-supplemented and control MLC were harvested and either tested for cytolytic reactivity in a standard 4-h ^{51}Cr-release assay or placed in TCGF-dependent culture. In some instances, effector cells were treated with anti-Thy-1 serum (1/20 dilution, mouse AKR-anti-C3H acites, No. 8301, Bionetics Laboratory Products, Litton Bionetics Inc., Kensington, Md.) and absorbed-rabbit complement (Cedar Lane Laboratories, London, Ontario, Canada) before use in ^{51}Cr-release assays.

Lymphocyte-Mediated Cytolysis (LMC) Assays. 4-h ^{51}Cr-release assays were conducted in 96-well, v-bottom, microplates (1S-MVC-96-TC, Linbro Chemical Co., New Haven, Conn.) using methodology previously described (5). The percentage of specific lysis was determined by using the following equation: % specific lysis = 100 × (experimental cpm − medium control cpm/maximum release cpm − medium control cpm). Data are displayed both graphically and in tabular form in terms of both lytic capacity (LC) and lytic efficiency (LE). LC is defined as the number of lytic units (LU)/25×10^6 effector cells. 1 LU is defined as the number of effector cells necessary to mediate 30% specific lysis. LE is defined as the percentage of specific lysis observed at an effector/target cell ratio of 100/1. Target cells used in LMC assays included the FBL-3(Hn) (H-2^b) murine leukemia cell line (5), the AKT-8 (H-2^k) thymoma cell line (obtained from Dr. Janet Hartley, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Md.) and the P815 (H-2^d) mastocytoma cell (obtained from Dr. H. Robson MacDonald, Ludwig Institute for Cancer Research, Lausanne, Switzerland). In addition to the above-detailed tumor targets, several normal mouse thymus cell populations were used as targets for nude mouse CTLL. The strains tested included: DBA-2 (H-2^d), DBA-1 (H-2^q), Ajax (H-2^a), and SJL (H-2^s).

Adsorption Of Anti-Thy-1 Serum by CTLL Cells. To test for cell surface expression of Thy-1 antigen, 100-μl aliquots of anti-Thy-1 serum (1/640) were incubated for 1 h at 37°C in the presence of increasing $\log_2$ concentrations of nude CTLL cells, normal murine CTLL cells, and normal C57Bl/6 thymocytes (concentrations ranged from 3×10^5 to 4×10^7 cells/ml). Adsorbed and control antisera were then tested for complement-mediated cytolysis directed against ^{51}Cr-labeled normal C57Bl/6 thymocytes using methodology previously described (5). The antiserum dilution (1/640) chosen for adsorption routinely generated between 70 and 100% complement-mediated cytolysis as assayed on normal mouse thymocytes. Results of adsorption experiments are expressed in terms of the percentage of inhibition of cytotoxicity observed after adsorption at a particular cell concentration.

Direct Membrane Immunofluorescence. Target cells to be assayed (nude CTLL or normal C57Bl/6 thymocytes) were adjusted to a concentration of between 5×10^7 and 2×10^8 cell/ml in cold Hanks' buffered salt solution (HBSS). 50 μl of cells was then incubated for 30 min at 4°C with an equal volume of either of two antisera: (*a*) fluorescein-conjugated, absorbed rabbit-anti-mouse (C3H) brain (anti-Thy-1, No. 8301-63, Bionetics Laboratory products or (*b*) fluorescein-conjugated goat-anti-mouse immunoglobulin (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.). Cells were washed twice with 5-ml vol of cold HBSS, resuspended in two–four drops of cold HBSS and observed with the aid of a Zeiss fluorescence microscope (Carl Zeiss, Inc., New York).

Results

Inability of Immature Prothymocytes to Produce TCGF. Because lymphoid organs from athymic nude mice represent populations of cells which contain appreciable numbers of immature prothymocytes, we questioned whether nude mouse lymphoid cells would produce TCGF in response to T-cell mitogenic stimulation. To assess TCGF production, both NIH and BALB/c nude mouse spleen, lymph node, and bone marrow cells (1×10^7 cells/ml) were cultured with a mitogenic dose of Con A (2.5 μg/ml). At 24-h culture intervals for each of 6 d, culture supernates were harvested and tested for TCGF activity. Spleen cells from normal BALB/c mice were cultured in an identical fashion. As detailed in Table I, nude mouse lymphoid cell populations were not capable of producing TCGF in response to Con A, whereas normal BALB/c spleen cells produced significant quantities of TCGF upon stimulation with Con A.

Response of Nude Mouse Lymphoid Populations To Con A and TCGF. To test whether nude mouse prothymocyte-containing cell populations were capable of responding to stimulation with Con A in the presence of TCGF, BALB/c and NIH nude mouse spleen, lymph node, and bone marrow cells were cultured in the presence of four different culture additives: (*a*) tissue culture medium, (*b*) Con A (2.5 μg/ml), (*c*) pp-TCGF and (*d*) pp-TCGF plus Con A (2.5 μg/ml). After 4 d of culture, cellular proliferation was assayed in terms of resultant ^{3}H-Tdr incorporation. As detailed in Fig. 1, all six responding cell types were capable of mounting a marked proliferative response to stimulation with Con A in the presence of TCGF. However, ^{3}H-Tdr incorporation by cultures treated with either Con A or pp-TCGF alone was no greater than the cellular proliferation witnessed in medium control cultures. The results detailed in Fig. 1 provide further evidence that after activation with Con A, lymphocyte proliferation is mediated solely via *in situ* production of, or exogenous supplementation with TCGF. Most importantly, the data detailed above show that, as was the case with normal mouse thymocytes,[2] nude mouse spleen, lymph node, and bone marrow cells are capable of mounting normal T-cell mitogen responses, as long as exogenous TCGF (which they are incapable of producing *in situ*) is supplied.

TCGF-Dependent In Vitro Generation of Nude Mouse Cytolytic Effector Cells. The observation that nude mouse spleen cells could respond to Con A in the presence of TCGF led us to question whether addition of TCGF to an identical cell population might be able to provoke a response to alloantigen as well. We have previously shown that the in vitro generation of alloantigen-specific, cytolytic effector T lymphocytes is thoroughly dependent upon alloantigen-provoked TCGF production (6). Furthermore, supplementation of cultures aimed at the generation of cytolytic effector cells with additional TCGF greatly increased both the quantity and efficiency of the cytolytic T-cells generated therein (6). Based on these observations, we hypothesized that perhaps addition of TCGF to nude mouse MLC might allow for the proliferative

TABLE I

TCGF Production by Normal and Nude Mouse Lymphoid Cell Populations

Responding culture*	TCGF production culture duration‡			
	24 h	48 h	72 h	96 h
BALB/c normal spleen cells	1.29	0.63	0.41	0.16
NIH nude mouse spleen cells	0.00	0.00	0.00	0.00
NIH nude mouse lymph node cells	0.00	0.00	0.00	0.00
NIH nude mouse bone marrow cells	0.00	0.00	0.00	0.00
BALB/c nude mouse spleen cells	0.00	0.00	0.00	0.00
BALB/c nude mouse lymph node cells	0.00	0.00	0.00	0.00
BALB/c nude mouse bone marrow cells	0.00	0.00	0.00	0.00

* 100 μl of cells (10^7 cells/ml) cultured with an equal volume of Con A (2.5 μg/ml) containing tissue culture medium.

‡ Units of activity contained in culture supernates.

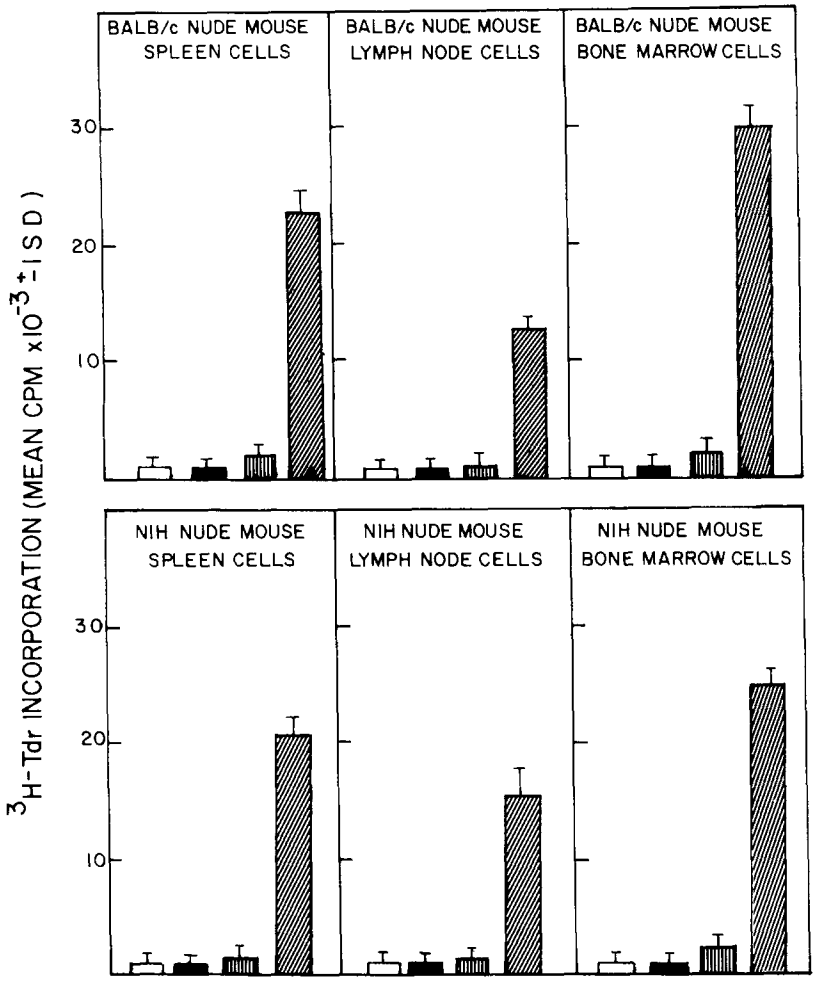

FIG. 1. ^{3}H-Tdr incorporation of 1 × 10^5 BALB/c and NIH nude mouse lymphocyte populations assayed after 96 h of culture in the presence of tissue culture medium (□); Con A (2.5 μg/ml, ■); pp-TCGF (▥); pp-TCGF plus Con A (2.5 μg/ml, ▨).

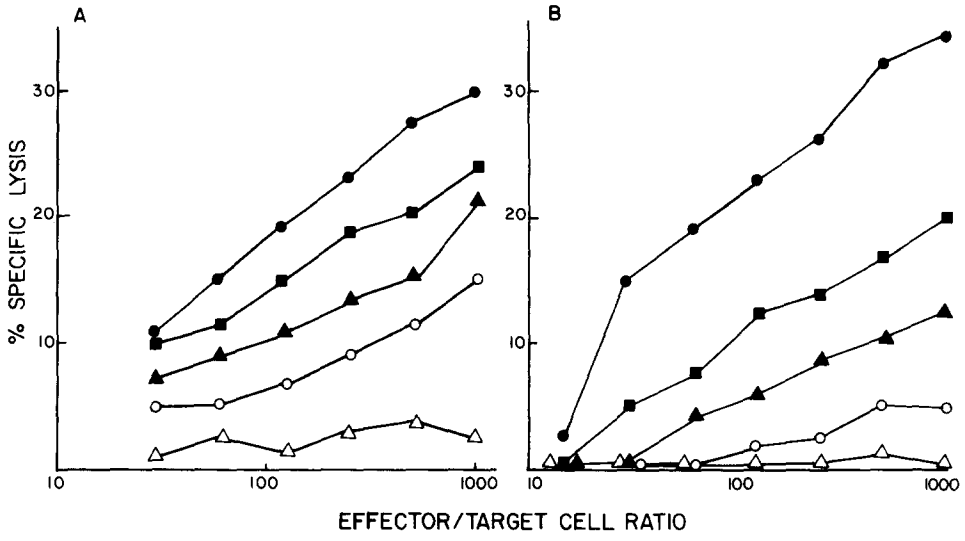

FIG. 2. Cytolytic reactivity of BALB/c (A) and NIH (B) nude mouse × C57Bl/6 MLC effector cells harvested from control cultures (△) and cultures supplemented with TCGF on day 0 (●), day 1 (■), day 2 (▲), and day 3 (○). Cytotoxicity directed against the FBL-3(Hn) (H-2^b) tumor target cell.

expansion of alloantigen-reactive responding cells to the extent where meaningful cytotoxic reactivity might be observed.

To test whether TCGF might initiate nude mouse lymphocyte-alloantigen reactivity, spleen cells from both NIH and BALB/c nude backgrounds were co-cultured in MLC with x-irradiated allogeneic C57Bl/6 spleen cells. On day 0–4, 10 ml of tissue culture medium was removed from one of five replicate flasks and replaced with 10 ml of TCGF. Viable effector cells harvested from 5-d control and TCGF-supplemented cultures, were then tested for cytolytic reactivity directed against a C57Bl/6 tumor target cell FBL-3(Hn) (H-2^b) in a standard 4-h ^{51}Cr-release assay. The ability of alloantigen (H-2^b)-sensitized nude mouse, splenic effector cells to lyse ^{51}Cr-labeled FBL-3(Hn) (H-2^b) target cells is shown in Fig. 2. Effector cells harvested from MLC supplemented with TCGF on day 0 mediated significant levels of cytotoxicity (30% specific lysis at an effector cell/target ratio of 400/1). TCGF supplementation on successively later days of culture resulted in the generation of effector cells which demonstrated a progressively weaker cytotoxic response. It is important to note that MLC stimulation in the absence of TCGF did not result in the generation of cytolytic effector cells. The results depicted in Fig. 2 confirmed that the addition of TCGF to nude mouse MLC resulted in the proliferation of alloantigen-reactive effector cells to the point where demonstrable cytotoxicity could be detected. Furthermore, the inability of nude mouse lymphoid cell populations to produce their own TCGF ensured that non-TCGF-supplemented MLC would be incapable of generating antigen-directed cytolytic effector cells.

Abrogation of Nude Mouse Effector Cell Cytotoxicity by Treatment with Anti-Thy-1 Serum and Complement. As previously detailed, only activated T cells have been shown to be capable of proliferating in response to TCGF (1–3, 6). The TCGF-dependent generation of nude mouse cytolytic effector cells, therefore, intimated that the cytotoxicity observed was mediated by cells derived from the T-cell lineage. A widely accepted

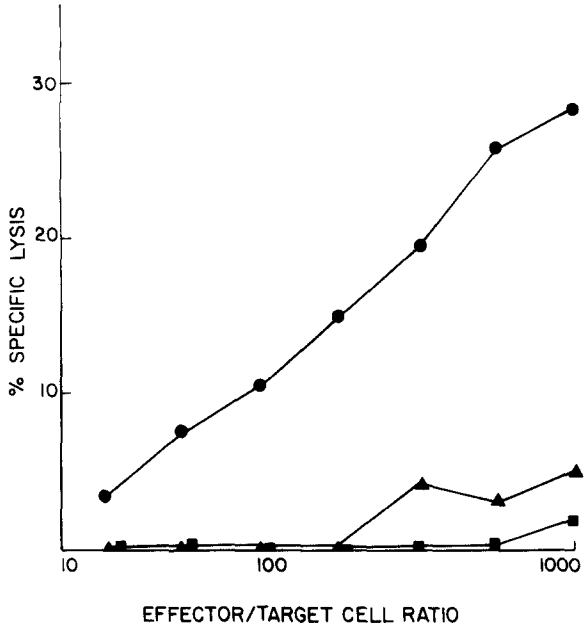

FIG. 3. Lysis of ^{51}Cr-labeled FBL-3(Hn) (H-2^b) tumor target cells by BALB/c nude mouse × C57BL/6 MLC effector cells: control effector cells (■); effector cells harvested from day-zero, TCGF-supplemented MLC (●); day-zero, TCGF-supplemented MLC effector cells treated with anti-Thy-1 serum and complement before use in LMC assay (▲).

demonstration of T-cell mediated cytotoxicity is the elimination of cytolytic reactivity after effector cell treatment with anti-Thy-1 serum and complement (7). The results of anti-Thy-1 serum and complement treatment of nude mouse cytolytic effector cells generated in TCGF-supplemented MLC are displayed in Fig. 3. Such treatment completely abrogated alloantigen-directed cytolysis. In fact, anti-Thy-1 serum-treated effector cells mediated little, if any, more cytolytic reactivity than did effector cells harvested from non-TCGF-supplemented MLC (2–4% lysis at an effector/target cell ratio of 1,000/1). Therefore, it appeared that immediate TCGF supplementation of nude mouse MLC resulted in the proliferative expansion of an allo-reactive prothymocyte (Thy-1-antigen-positive) population which was in turn capable of mediating significant alloantigen-directed cytolytic reactivity.

Creation of Alloantigen-Specific Nude Mouse CTLL. We have demonstrated that both mouse, and human, antigen-specific, cytolytic T cells may be maintained indefinitely in a TCGF-dependent state of exponential proliferation (1, 2). Indeed, two murine, Thy-1-antigen-positive CTLL have remained in culture in the presence of TCGF for over 2.5 yr. The demonstration that cytolytic effector cells generated in TCGF-supplemented nude mouse MLC were also Thy-1-antigen-positive, provided some basis for anticipating that perhaps the continuous culture of nude mouse cytolytic effector cells would also be possible.

In hopes of creating a nude mouse CTLL, effector cells harvested from nude mouse MLC (BALB/c nu/nu vs. C57Bl/6) were seeded in replicate flasks in a solution containing 50% supplemented Click's medium and 50% TCGF. Initial cultures were seeded at concentrations well below 3×10^5 cells/ml to ensure that effector cells were

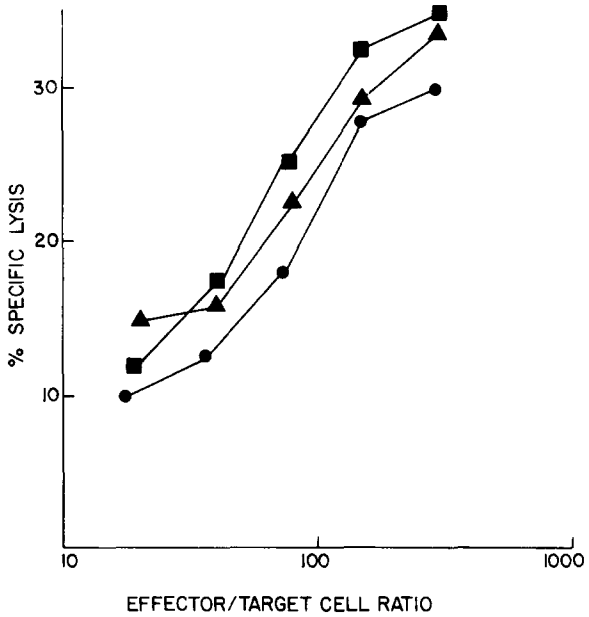

FIG. 4. Cytolysis of FBL-3(Hn) (H-2^b) tumor target cells by BALB/c × C57Bl/6 nude CTLL cells after 3 (●), 7 (■), and 11 (▲) wk of TCGF-dependent culture.

always in a situation of TCGF excess. Throughout the 1st 3 wk of culture, cells growing in suspension were routinely layered over a solution of Ficoll-Hypaque (Ficoll, Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J., Hypaque, Radiopaque Media, Winthrop Laboratories, New York) (2) before subculturing in fresh Click's medium/TCGF, to ensure that subcultured cells contained a minimal amount of cell debris. It is our belief that debris present in early stages of CTLL cell culture are the result of the eventual degradation of non-TCGF-responsive cells. In the case of TCGF-dependent culture of nude mouse MLC effector cells, cell debris present in initial cultures were particularly acute, presumably due to a large number of nonlytic cells (B cells, macrophages).

On the protocol of subculturing to 2 × 10^4 cells/ml, once cells reached a density of 2–4 × 10^5 cells/ml, nude mouse MLC effector cells have remained in a TCGF-dependent state of exponential proliferation for over 14 wk. The cells have the same morphological and growth characteristics (saturation density of 2–4 × 10^5 cells/ml, doubling time: every 18–30 h) as previously described CTLL (1, 2, 8–10). Deprivation of TCGF leads to irreversible cell damage and complete death of nude CTLL cultures within 24 h.

Throughout their culture, nude mouse CTLL have continued to mediate the in vitro cytolysis of allogeneic, FBL-3(Hn) (H-2^b), leukemia cells as displayed in Fig. 4. Nude mouse CTLL harvested from 3-, 7-, and 11-wk-old TCGF-dependent cultures demonstrated almost identical in vitro lytic reactivity directed against the FBL-3(Hn) (H-2^b) target cell. It is important to note that nude mouse CTLL mediated 30% specific lysis of the FBL-3(Hn) (H-2^b) target cell at an effector/target cell ratio of ≦150/1; a considerable increase in lytic efficiency as compared to effector cells harvested directly after TCGF-supplemented MLC stimulation (Figs. 2 and 3). As

TABLE II

Specificity of Cytotoxicity Mediated by Nude CTLL Cells

Age of CTLL culture	Cytotoxicity directed against							
	Tumor target cells			Thymocyte target cells				
	FBL-3(Hn) (H-2^b)	AKT-8 (H-2^k)	P815 (H-2^d)	C57Bl/6 (H-2^b)	DBA/2 (H-2^d)	DBA/1 (H-2^q)	SJL (H-2^s)	AJAX (H-2^a)
wk								
8	390/37*	0.8/3	4.1/6	227/26	<0.1/3	0.7/4	<0.1/3	0.4/2
12	250/31	1.1/2	5.0/8	116/21	0.3/6	<0.1/2	<0.1/1	<0.1/0

* Expressed at LC/LE. LE = LU/25 × 10^6 nude CTLL cells. LC is the percentage of specific lysis observed at an effector/target cell ratio of 100/1.

mentioned above, we have hypothesized that the observed increase in lytic effect after prolonged culture may be due to a gradual elimination of non-TCGF-responsive cells and a reciprocal enrichment of the culture population for TCGF-dependent CTLL.

The cytolysis mediated by nude CTLL cells was observed to be alloantigen-(H-2^b)-specific. As detailed in Table II, nude CTLL harvested after 8 and 12 wk of long-term culture were capable of effecting only the lysis of H-2^b target cells (FBL-3(Hn) and C57Bl/6 thymocytes). DBA-2 (H-2^d) thymocyte target populations as well as the P815 (H-2^d) tumor target cell were unaffected. In addition, nude CTLL mediated no cytolytic reactivity whatsoever against the third party tumor target cell, AKT8 (H-2^k) nor were (H-2^b)-reactive nude CTLL capable of killing several third party thymocyte target populations: DBA-1 (H-2^q), SJL (H-2^s), and Ajax (H-2^a).

Throughout their long-term culture, nude CTLL have remained Thy-1-antigen-positive, surface-immunoglobulin-negative, and negative by histochemical stains for specific and nonspecific esterases. Cell surface expression of Thy-1 antigen was determined by two distinct assays: (*a*) adsorption of anti-Thy-1 serum activity and (*b*) direct membrane immunofluorescence. In adsorption tests, increasing numbers of nude CTLL cells were used to adsorb anti-Thy-1 serum cytotoxic reactivity as tested on ^{51}Cr-labeled normal C57Bl/6 thymocytes in standard antibody-dependent, complement-mediated cytolysis assays. As detailed in Fig. 5, 12-wk-old nude CTLL cells were quite efficient in effecting the concentration-dependent adsorption of anti-Thy-1 serum reactivity (50% inhibition of cytotoxicity after adsorption with 2.5 × 10^5 cells). Furthermore, the ability of nude CTLL to adsorb anti-Thy-1 serum activity was essentially identical to that demonstrated by normal murine CTLL cells or normal C57Bl/6 thymocytes.

The results of direct membrane immunofluorescence tests conducted on both normal C57Bl/6 thymocytes and 14-wk-old nude CTLL cells are shown in Fig. 6. Both nude CTLL (Fig. 6A) and normal thymocyte populations (Fig. 6B) were found to be 95–100% Thy-1-antigen-positive in tests using fluorescein-conjugated, absorbed rabbit-anti-mouse brain antiserum. With identical direct immunofluorescence techniques and fluorescein-conjugated goat-anti-mouse immunoglobulin, nude CTLL cells were found to be 100% negative for surface-bound immunoglobulin (Fig. 6C). Therefore, as determined by three separate assays: (*a*) inhibition of cell-mediated cytotoxicity by effector cell treatment with anti-Thy-1 serum and complement; (*b*) adsorption of cytolytic reactivity from anti-Thy-1 serum; and (*c*) direct membrane

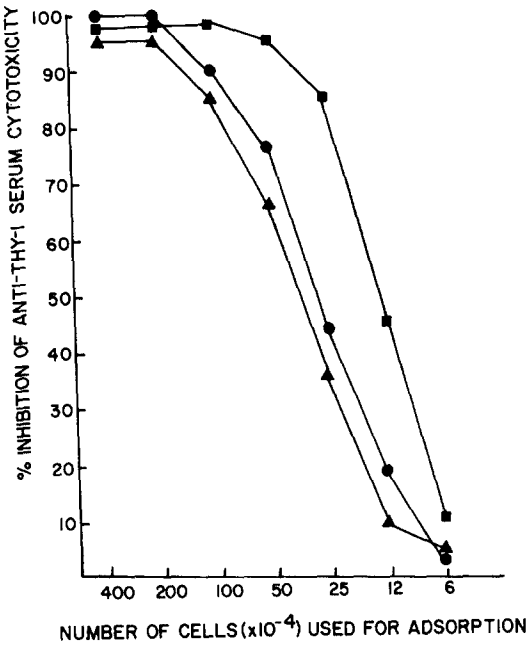

FIG. 5. Concentration-dependent adsorption of anti-Thy-1 serum cytotoxic reactivity by nude mouse CTLL cells (▲), normal murine CTLL cells (●), and C57Bl/6 thymocytes (■). 100 μl of cells incubated for 1 h at 37°C with an equal volume of anti-Thy-1 serum before testing for complement-mediated cytolysis of normal thymocytes. Resultant serum dilution (1/640) routinely promoted 80–100% specific lysis of thymocyte targets.

immunofluorescence, it appears that alloantigen-specific, cytotoxic effector cells generated in TCGF-supplemented nude mouse MLC and maintained in a TCGF-dependent state of exponential proliferation are Thy-1-antigen-positive cytotoxic T lymphocytes.

Discussion

The findings detailed in this report have shown that dual stimulation of nude mouse spleen cells with both alloantigen and TCGF allows for the proliferative expansion of allo-reactive, Thy-1 antigen-positive, cytotoxic lymphocytes. Furthermore, lytic specificity conferred by alloantigen stimulation is retained by nude CTLL throughout 3 mo of subsequent TCGF-dependent culture. These results, compounded with the observation that TCGF also allows for normal T-cell mitogen stimulation of nude mouse spleen, lymph node, and bone marrow cells, may have important ramifications as to our present thinking regarding several aspects of T-cell differentiation; most notably, the functional potential of pre-thymocytes, the role of the thymus in directing T-cell maturation, and, finally, possible mechanisms for overcoming pathological disease states stemming from thymic deficiencies.

Several other investigators have previously reported that nude mouse spleen cells, when used as responding lymphocyte populations in MLC, were not capable of effecting the in vitro cytolysis of ^{51}Cr-labeled allogeneic target cells, even when tested at killer/target cell ratios as high as 400/1 (11, 12). These investigators concluded

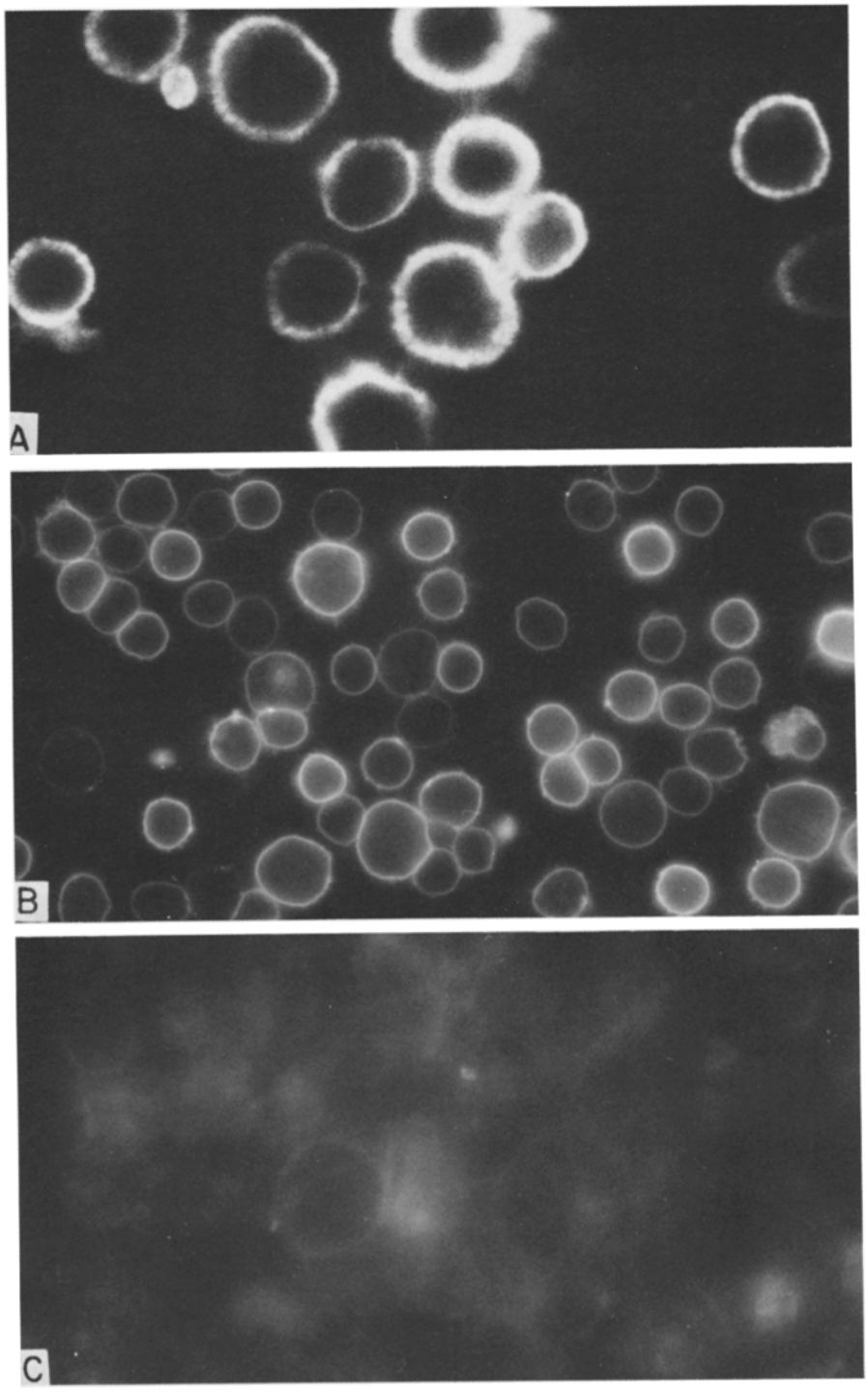

FIG. 6. Cell surface labeling of nude CTLL cells (A) and normal C57Bl/6 thymocytes (B) via direct immunofluorescence with fluorescein-conjugated anti-Thy-1 serum. Cell surface labeling of nude CTLL cells with fluorescein-conjugated goat-anti-mouse immunoglobulin (C). Approximately ×3,200.

that the lack of cytotoxicity mediated by nude mouse MLC-activated spleen cells was due to a lack of cytotoxic precursors. The experimentation detailed above (Figs. 2 and 3) suggests that the inability of the nude mouse to manage an effective MLC reaction is not due to a dearth of appropriate reactive precursors, but is instead due to the lack of cells which are capable of producing TCGF (Table I). As we have previously described, the in vitro generation of cytolytic T-cells is thoroughly dependent upon the ability of responding lymphocyte populations to produce TCGF as triggered by alloantigen sensitization (6). Because nude mouse cell populations were unable to produce TCGF, it followed that cells from nude mice would be unable to generate cytolytic cells in classical MLC. However, when TCGF was provided exogenously, nude mouse splenic responder cells were capable of proliferating in response to alloantigen stimulation to the point where demonstrable in vitro cytolysis was observed. It should be noted that the level of cytolysis observed (30% specific lysis at an effector cell/target ratio of 400:1), was less than the efficiency of lysis we routinely observe when normal spleen cells were used as responder cells in an MLC (100% specific lysis at an effector cell/target ratio of 100:1). It remains to be investigated whether this difference is due to relatively fewer alloreactive nude precursor T cells or whether additional factors (perhaps thymic humoral factors) are necessary to effect the maturation of all precursor T cells to a fully TCGF-responsive state. Nonetheless, these results suggested that nude mice contained alloreactive precursor T cells which, when provided with antigen and exogenous TCGF, were capable of mediating a specific immunologic function previously ascribed only to normal, mature, T lymphocytes.

Considerable evidence has been presented detailing the fact that athymic nude mice contain thymic precursor cells which can function to reconstitute lethally irradiated mice (13). Chromosome analyses have shown without question that reconstitution in such cases is due to the evolution of a new T-lymphoid system stemming from the nude donor rather than from some radioresistant subpopulation of the recipient (14). Conversely, nude mice themselves can be reconstituted to normal T-cell function after implantation of a thymus graft (15) or thymic epithelial tissue (16). In both types of studies, the concensus has been that restoration of T-dependent function was the result of some type of interaction between thymus tissue or tissue products and nude mouse precursor T cells. Such an interaction might involve the actual training and differentiation of prothymocytes into antigen-reactive cells which, when later exposed to antigen, are capable of responding in the manner imparted to them during exposure to thymic influence.

Of particular interest with regard to the influence of the thymus on the immunologic capacity of immature T cells, was the recent report by Irle et al. (17). These investigators found that immature thymocytes were capable of mediating normal immunologic, in vitro, responses provided cultures were supplemented with medium conditioned by mitogen-stimulated peripheral T lymphocytes. On the basis of these observations, it was postulated that the thymus was responsible for two steps in the differentiation of immature precursors into mature, immunologically reactive T lymphocytes: (*a*) the acquisition of specific mitogen and antigen receptors thought to occur during cell division in the thymic cortex, and (*b*) the development of the capacity to respond to antigen-receptor triggering by proliferation in the absence of mitogenic factors released by activated lymphoid cells. Irle et al. (17) further concluded that mature peripheral T cells did not require extracellular factors to mediate

proliferation in response to mitogenic or antigenic stimulation.

The experimentation described herein, as well as our previous observations (1–3, 6),[2] provides evidence that immature, nude mouse prothymocytes (in the absence of thymic influence) possess mitogen and alloantigen responsiveness, and that prothymocytes, as well as peripheral mature T-lymphocytes, require a second proliferative signal to respond to mitogen/antigen sensitization. Other investigators have described similar studies involving the provocation of T-cell function in the nude mouse which supports the *de novo* presence of antigen-specific pre-T-cells capable of functioning without prior exposure to thymus tissue or tissue products. In fact, these studies, when interpreted with the knowledge of the pivotal role that TCGF plays in the proliferation of activated T lymphocytes, lend further evidence to our contention that the specific influence mediated by a thymus graft in the reconstitution of nude mouse T-lymphoid function, is to provide for the maturation of TCGF-producing cells.

For example, Schimpl and Wecker (18) as well as Kindred and Corley (19) have shown that treatment of nude mice with tissue-culture supernates derived from normal MLC restored the ability to produce antigen-specific antibody directed against sheep erythrocytes (SRBC) or alloantigen. Both sets of investigators suggested that the mode of action of "helper factors" present in the MLC supernate was directly on B cells reactive to the particular antigen involved. However, in light of our findings that MLC as well as mixed tumor lymphocyte cultures produced copius quantities of TCGF (6), it is plausible to hypothesize that the TCGF present in MLC supernates together with antigen presentation allowed for the proliferation of antigen-specific helper pre-T-cells already present in the nude mouse. The TCGF-dependent proliferation of these cells then allowed for specific B-cell activation and antibody production.

Such a hypothesis is similar to that offered by Farrar et al. (20) to explain results they obtained detailing the ability of helper peak 1 (HP-1 isolated from MLC supernates) to reconstitute anti-SRBC reactivity by T-cell deficient spleen cell populations. As opposed to implicating a direct effect of HP-1 on antibody-producing B cells, Farrar et al. (20) acknowledged the possibility that helper molecules might be involved in the activation of pre-T-cells which in turn provided the putative antigen-specific helper signal. The observation that rat pp-TCGF has a mol wt of ≅14,000 daltons (similar to the 10,000–15,000 dalton mol wt described for HP-1)[3] lends further support to the hypothesis that MLC supernate-dependent reconstitution of T-dependent, antigen-directed antibody production might have been due to the effects of a TCGF-dependent proliferation of pre-helper T cells already present in T-cell-deficient populations.

The TCGF-driven proliferation of antigen-reactive nude mouse pre-T-cells also provides a means for explaining the results of Piguet and Vassalli (21) who recently reported that intravenous injection of nude mice with allogeneic or xenogeneic purified T-cell populations resulted in the ability of the T-cell treated mouse to reject a skin graft syngeneic to the T-cell donor (via production of graft-specific cytotoxic antibody). These investigators suggested that the ability of nude mice to respond to a foreign graft after treatment with graft strain T cells, represented a unique form of T-B-cell cooperation across both histocompatibility and species barriers in which the grafted T cells recognized nude mouse histocompatibility antigens and in some manner provided a means of helping nude mouse B cells to make antibody against

the graft. Once more, the knowledge that TCGF is produced by alloantigen stimulation provides us with a mechanism to explain the above-mentioned data. Donor cells, upon recognizing nude mouse histocompatibility antigens as foreign, produced TCGF which, in turn, allowed for the proliferation of host, graft-antigen-specific, helper pre-T-cells whose action resulted in syngeneic T-B-cell cooperation eventually leading to allo- or xeno-graft rejection. The nude mouse, incapable of producing its own TCGF, was the beneficiary of graft vs. host-generated TCGF. Therefore, it is conceivable that once more, the TCGF-dependent proliferation of already existent antigen-reactive nude mouse pre-T-cells triggered the demonstration of competent T-cell immune function.

The hypothesis that antigen-specific pre-T-cells exist in the nude mouse and are capable of contributing in cell-mediated reactions without additional thymic influence should not be construed as incongruous with the repeated demonstration that exposure of T-cell deficient lymphoid populations to thymic influences either in vitro or in vivo can result in total reconstitution of T-cell function. In fact, co-culture of T-cell-deficient lymphocyte populations or nude mouse lymphoid cell populations with thymic epithelial cells (22–24), supernates from thymic epithelial cells (24, 25), or thymic extracts (26–28) (in particular, thymosin [29, 30]), has been shown to result in several manifestations of normal T-cell function, including: acquisition of T-cell surface markers (31–33); reconstitution of normal proliferative responses to T-cell mitogens (22); and reconstitution of in vitro responsiveness to T-dependent antigens such as SRBC (34), dinitrophenol-protein conjugates, and alloantigen (35). The data presented in this report, in particular, the inability of nude mouse lymphoid cell populations to produce TCGF and still respond to both T-cell mitogen and alloantigen provided exogenous TCGF was present, lead us to hypothesize that the influence of the thymus in the above-cited T-cell function reconstitution experiments was to program the differentiation of prothymocytes into mature T cells capable of producing TCGF. Indeed, it is our belief that a major function of the thymus as a site of T-cell differentiation is to influence the maturation of T lymphocytes capable of cooperating in the production of TCGF.

It should be noted that the pp-TCGF used in the experimentation described in this report might contain more than one molecular entity. In experimentation to be reported elsewhere, we have found that the TCGF activity obtained from DEAE-cellulose chromatography elutes as a single protein moiety after Bio-Gel-p30 (Bio-Rad Laboratories, Richmond, Calif.) gel-exclusion chromatography. Furthermore, active fractions pooled after DEAE chromatography migrate as a single peak of activity on analytical isoelectric focusing columns (isoelectric point = 5.65).[3] Although these results imply that the TCGF activity present in pp-TCGF (obtained via DEAE chromatography) resides in a single molecular entity, it remains possible that additional factors with similar molecular characteristics (present in pp-TCGF preparations) might also be involved in effecting the results we have described.

The biologic activity that distinguishes TCGF from other previously described mitogenic factors (see 36 for review) is its ability to initiate and sustain the continuous proliferation of activated T cells. TCGF production requires mature lymphocytes (either cortisol-resistant thymocytes or peripheral T cells)[2] and is elicited as a consequence of T-cell mitogen or antigen stimulation (3). Furthermore, only activated T cells absorb TCGF activity and respond by continuous proliferation. T-cells which

have not been exposed to mitogen or antigen, as well as activated B lymphocytes, fail to absorb TCGF activity and do not proliferate in response to TCGF (4). Therefore, the weight of the evidence indicates that TCGF acts as a second signal effecting the proliferation of mature activated T cells and that prothymocyte populations respond in a similar manner after mitogen or antigen sensitization in the presence of TCGF.

Whether or not the function of the thymus in T-cell differentiation is mediated solely by effecting the maturation of TCGF-producing cells or is intimately associated with conferring antigen-specificity upon nondirected prothymocytes, the fact that TCGF restores normal T-cell responses to nude mouse spleen cells, suggests that TCGF may be capable of alleviating a great deal of the T-cell immunodeficiency presented by the nude mouse. The observations that: (*a*) nude mouse spleen cells generate cytolytic effector cells after TCGF-supplemented MLC stimulation and (*b*), antigen-specific nude mouse killer T cells can be maintained in culture indefinitely in the presence of TCGF, provide evidence that TCGF-treatment itself may lead to the development of a new modality for short-circuiting and treating T-cell immune deficiencies. It is our hope that continued studies of the effect of TCGF on nude mouse immune reactivity compounded with further investigation regarding the role of TCGF in thymus-directed T-cell maturation and differentiation, will provide additional insight into the regulation of both normal and deficient T-cell immune reactivity.

Summary

In addition to allowing for the long-term culture of both murine and human cytolytic T lymphocytes, T-cell growth factor (TCGF) functions as the key proliferation-inducing second signal in both T-cell antigen sensitization and mitogenesis. The observation that thymocytes responded normally to T-cell mitogens in the presence of TCGF, prompted the investigation of the effect of TCGF on nude mouse lymphocyte responses in vitro. We found that spleen, lymph node, and bone marrow cells, isolated from nude mice, were incapable of producing TCGF yet responded normally to T-cell mitogen sensitization provided stimulation was conducted in the presence of TCGF. Nude mouse spleen cells were also capable of responding to alloantigen sensitization in mixed lymphocyte cultures (MLC) conducted in the presence of TCGF. Thy-1 antigen-positive cells harvested from TCGF-supplemented nude mouse MLC effectively mediated the cytolysis of alloantigen-specific target cells as tested in standard ^{51}Cr-release assays. Cytolytic nude mouse effector cells have remained in TCGF-dependent culture for over 3 mo during which they have continued to mediate significant levels of alloantigen-specific cytolytic reactivity. These results suggest that prothymocytes present in nude mice are capable of responding to immunologic stimuli by differentiating, in vitro, into cytolytic T lymphocytes and that furthermore, a major function of the thymus may be to effect the maturation of TCGF-producing cells.

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