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Research Article

The ability of the monoclonal antibody directed at the T3 antigen (anti-T3) to induce cytolytic activity was investigated since several agents that can activate T cells induce the acquisition of cytolytic activity in a variety of test systems. Pretreatment of human alloimmune memory cells, generated in primary long-term mixed lymphocyte cultures, with anti-T3 resulted in the induction of statistically significant specific secondary cytolytic activity and natural killer (NK) cell-like activity. No such augmentation or induction of cytolytic activity was found with anti-T3 pretreatment when syngeneic cells or inappropriate allogeneic cells (HLA-A, B antigens different from the original priming stimulus) were used as target cells and pretreatment of memory cells with anti-T4 or anti-T8 did not induce cytolytic activity to allogeneic or syngeneic target cells. Differential effects were observed when anti-T3 was added to the cytotoxicity assay in which anti-T3 pretreated alloimmune memory cells were effectors. The addition of anti-T3 to the assay prior to the introduction of target cells resulted in 39 +/- 8% inhibition of specific secondary cytolytic activity and only 5 +/- 8% inhibition of NK cell activity. NK cell activity mediated by large granular lymphocyte-enriched fraction of peripheral blood mononuclear cells (PBM) obtained from normal individuals was significantly augmented by anti-T3 when NK-sensitive cell lines MOLT-4 or K-562 were used as target cells. This augmentation in NK […]

Induction of Cytolytic Activity by Anti-T3 Monoclonal Antibody

Activation of Alloimmune Memory Cells and Natural Killer Cells from Normal and Immunodeficient Individuals

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Abstract. The ability of the monoclonal antibody directed at the T3 antigen (anti-T3) to induce cytolytic activity was investigated since several agents that can activate T cells induce the acquisition of cytolytic activity in a variety of test systems. Pretreatment of human alloimmune memory cells, generated in primary long-term mixed lymphocyte cultures, with anti-T3 resulted in the induction of statistically significant specific secondary cytolytic activity and natural killer (NK) cell-like activity. No such augmentation or induction of cytolytic activity was found with anti-T3 pretreatment when syngeneic cells or inappropriate allogeneic cells (HLA-A, B antigens different from the original priming stimulus) were used as target cells and pretreatment of memory cells with anti-T4 or anti-T8 did not induce cytolytic activity to allogeneic or syngeneic target cells.

Differential effects were observed when anti-T3 was added to the cytotoxicity assay in which anti-T3 pretreated alloimmune memory cells were effectors. The addition of anti-T3 to the assay prior to the introduction of target cells resulted in 39±8% inhibition of specific secondary cytolytic activity and only 5±8% inhibition of NK cell activity.

NK cell activity mediated by large granular lymphocyte-enriched fraction of peripheral blood mononuclear cells (PBM) obtained from normal individuals was significantly augmented by anti-T3 when NK-sensitive cell lines MOLT-4 or K-562 were used as target cells. This augmentation in NK cell activity was not associated with nonspecific cytotoxicity to syngeneic or allogeneic PBM, and anti-T3 failed to activate the LGL fraction depleted of T cells. The monoclonal antibodies, anti-T4 or anti-T8, did not increase NK cell activity.

NK cell activity mediated by PBM from eight immunodeficient individuals (four with acquired immunodeficiency syndrome and four with renal allografts) was also significantly augmented by anti-T3 pretreatment. Our findings, in addition to providing a rationale for the frequent occurrence of re-rejection episodes in renal graft recipients treated with anti-T3, suggest that anti-T3 might be utilized to enhance the cytotoxic armamentarium of immunodeficient patients.

Introduction

The monoclonal antibody (MoAb),[1] anti-T3, directed at a 20,000-molecular weight glycoprotein expressed on virtually all postthymic T cells, is currently used as an immunosuppressive agent in organ graft recipients (1–3). The ability of anti-T3 to inhibit antigen-specific T cell proliferative responses and generation of cytotoxic T cells (CTL) in mixed lymphocyte cultures (MLC) (4, 5), as well as its ability to inhibit CTL activity at the effector level (5, 6), provided the rationale for the use of this agent as an immunosuppressant. Additional studies, however, have revealed several properties of anti-T3

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1. *Abbreviations used in this paper:* AIDS, acquired immunodeficiency syndrome; CM, complete medium; Con A, concanavalin A; CTL, cytoxic T cells; IL-2, interleukin-2; LGL, large granular lymphocyte-enriched fractions of PBM; MLC, mixed lymphocyte cultures; MoAb, monoclonal antibody; NHS, normal human serum; NK, natural killer; PBM, peripheral blood mononuclear cells; PHA, phytohemagglutin; SCR, specific ^{51}Chromium release.

that might compromise the immunosuppressive potential of anti-T3 MoAb. This includes the ability of anti-T3 to activate human peripheral blood mononuclear cells (PBM) (7), enhance responsiveness to interleukin-2 (IL-2) (1), and induce lymphokine production (8–10). Because several polyclonal activators, including oxidizing mitogens, can activate alloimmune memory cells resulting in their acquisition of secondary cytolytic activity in the absence of original priming stimulus (11), and because IL-2 and interferon can augment or induce cytolytic activity (12, 13), we reasoned that anti-T3, despite the previous findings of inhibition of cytolytic activity by anti-T3 (4–6), can induce or augment cytolytic activity.

In the present communication, we report that anti-T3 can: (*a*) activate alloimmune memory cells (memory cells) resulting in their acquisition of specific secondary cytolytic activity, (*b*) augment natural killer (NK) cell activity, and (*c*) partially correct the impaired NK cell activity found with PBM obtained from patients with acquired immunodeficiency syndrome (AIDS) and from renal allograft recipients.

Methods

Monoclonal antibodies. Azide-free lyophilized monoclonal antibodies directed at T3, T4, and T8 antigen were graciously provided by Ortho Diagnostic Systems, Inc., Raritan, NJ. The antibodies were initially reconstituted with 1 ml of distilled water and then adjusted to desired concentrations in RPMI 1640 medium (MA Bioproducts, Walkersville, MD).

Fluorescein-conjugated anti-human Leu-7 and anti-human Leu-11a were purchased from Beckton-Dickinson Monolonal Center Inc., Mountain View, CA, and fluorescein-conjugated monoclonal antibodies, anti-human T cells (Ortho-mune OKT3), anti-human inducer/helper T cells (Ortho-mune OKT4), and anti-human T cell (Ortho-mune OKT11) were purchased from Ortho Diagnostics Systems, Inc. All immunofluorescence analyses to identify cell surface antigens were carried out using an Ortho Spectrum III Laser Flow Cytometry System (14).

Activation of cells with anti-T3. The monoclonal antibody, anti-T3, was tested for its ability to activate alloimmune memory cells and large granular lymphocyte-enriched fractions (LGL) of PBM.

Memory cells were generated in primary long-term mixed lymphocyte culture (MLC) as described previously (11). The responder cells (memory cells) were collected 10–14 d after initiation of the primary long-term (MLC) and washed twice with RPMI 1640 medium (MA Bioproducts) containing 100 U/ml penicillin, 100 μg/ml streptomycin, 25 mM Hepes buffer, and 5% heat-inactivated pooled normal human serum (complete medium [CM]). The memory cells (1×10^6 cells/ml) were co-cultured with equal numbers of irradiated (3000 rad, Cesium source) syngeneic cells or allogeneic cells (original sensitizing stimulus) or with anti-T3. Proliferation was determined by measuring [^{3}H]thymidine (New England Nuclear, Boston, MA; specific activity, 2 Ci/mmol) incorporation during 48–64 h of culture. To determine cytolytic activity, the memory cells were retrieved after 3 d of culture, washed twice, resuspended in CM, and used as effector cells in the ^{51}Cr-release cytotoxicity assay (11).

LGL were isolated by a modification of the technique of Timonen and Saksela (15). In brief, Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) was prepared isoosmotic with ten times concentrated Hanks' balanced salt solution (GIBCO, Grand Island, NY) to produce a 90% mixture. The discontinuous gradient was assembled using 2 ml, 90%; 2 ml, 62%; 3 ml, 53%; 3 ml, 44%; and 2 ml, 36% mixtures as successive layers in a 15-ml conical centrifuge tube (Falcon 2095, Falcon Labware, Oxnard, CA). Nonadherent fractions of PBM (5×10^7 cells in 3 ml of CM), depleted of adherent cells by passage over a Sephadex G-10 (Pharmacia) column (16), were placed on top of the gradient and centrifuged (1,000 *g*) for 25 min at 20°C. The LGL-enriched fraction was recovered at the 44:53% interface. The LGL fraction was washed twice by centrifugation for 10 min at 300 *g*. Almost all NK cell activity was found in the LGL-enriched fraction and this fraction contained 40–60% cells reacting with anti-human Leu-7 or anti-human Leu-11a and 20–30% cells reacting with anti-T3 or anti-T11. LGL fractions were contaminated with <1% monocytes or polymorphonuclear leukocytes as determined by nonspecific esterase staining (17) or by scoring LGL in a Neubaur counting chamber (American Optical Corp., Buffalo, NY) covered with an acridine orange-coated coverslip with simultaneous ultraviolet (UV) light and phase-contrast illumination (18).

In some experiments, prior to Percoll density gradient centrifugation to obtain LGL, nonadherent fractions of PBM were depleted of T cells by sheep erythrocyte rosetting technique as previously described (16) or by the panning technique of Wysocki and Sato (19). Depletion of T cells by sheep erythrocyte-rosetting technique was accomplished by mixing 1-ml aliquots of neuraminidase-treated sheep erythrocytes and pooled normal human serum (NHS) with 1-ml aliquot of nonadherent PBM ($2–3 \times 10^7$ cells/ml). This mixture was centrifuged at 300 *g* at 4°C for 10 min, followed by incubation at 4°C for 60 min. The pellet was gently resuspended and overlayered onto 4 ml Ficoll-Hypaque (Pharmacia Fine Chemicals), and centrifuged at 500 *g* at 4°C for 30 min. The T cell-depleted fraction (nonrosetting) was collected as the buffy layer at the Ficoll:medium interface, and the T cell-depleted nonadherent fraction of PBM was washed twice before Percoll density gradient centrifugation.

Depletion of T3 antigen-positive cells by the panning technique was accomplished by first sensitizing nonadherent fractions of PBM with the monoclonal antibody anti-T3. The cells were incubated with 500 ng/ml for 40 min at 4°C. The antibody-treated cells were then diluted to 25×10^6 cells/3 ml in phosphate-buffered saline (PBS) with 5% NHS. 3-ml aliquots were placed into Fisher bacteriological petri dishes (100 mm diam, No. 8-757-12, Fisher Scientific Co., Pittsburgh, PA) coated with affinity-purified fraction of goat anti-mouse IgG (Cappel Laboratories, Cochranville, PA) and incubated for 70 min at 4°C. T3 antigen negative cells were harvested as the nonadherent cells aspirated in the supernatant following such incubation. The cells were then washed twice and LGL from these fractions were obtained as described earlier.

To determine the effect of monoclonal antibodies on NK cell activity, LGL were cultured for 16 h with specified concentrations of antibodies at 37°C in a 95% air/5% CO_2 humidified atmosphere. At the end of incubation, the cells were retrieved, washed twice, and suspended in CM before use as effector cells in the ^{51}Cr-release cytotoxicity assay (11).

^{51}Cr-Release cytotoxicity assays. The ^{51}Cr-release cytotoxicity assays were performed with slight modification of a previously described technique (11). In brief, pretreated or untreated PBM or memory cells or LGL suspended in CM functioned as effector cells. The following were used as target cells in the cytotoxicity assay. The T lymphoblastoid cell line, MOLT-4 (N.I.G.M.S. Human Genetic Mutant Cell Repository, Institute for Medical Research, Camden, NJ), and the erythromyeloid

cell line, K-562 (originally provided by Dr. Y. B. Kim, Sloan Kettering Institute, Rye, NY), were maintained as suspension cultures in our laboratory and used as NK-sensitive targets. PBM, freshly prepared by Ficoll-Hypaque gradient centrifugation of heparinized venous blood, were also used as target cells. The PBM were neither modified nor activated with lectins before their utilization as target cells. HLA-A, B antigens expressed on PBM were identified using the standard microlymphocytotoxicity assay. The target cells were labeled with ^{51}Cr by incubating 2×10^6 cells with 300 μCi of Na_2CrO_4 for 1 h at 37°C. After washing twice, the cells were adjusted to 1×10^5 cells/ml. The effector and target cells were incubated at various ratios in round-bottomed microtiter plates (Nunc, 2-62170, Vanguard International, Inc., Neptune, NJ) of 200-μl well volume for 3–4 h at 37°C. At the end of incubation, the plates were centrifuged (500 $g \times 10$ min) and 100 μl of supernatants were aspirated and counted in a liquid scintillation counter (Packard Instruments Co., Inc., Downers Grove, IL). Percent specific chromium release (SCR) was calculated using the following formula: $(ER - SR) \div (MR - SR) \times 100$, where *ER* is experimental release (counts per minute found with effector and target cells), *SR* is spontaneous release (counts per minute found with target cells alone), and *MR* is maximal release (counts per minute found with target cells incubated with 1% Triton-X in distilled water). Spontaneous release for the target cells used was in the range of 5 to 15% after 3–4 h of incubation and the maximum release was in the range of 85 to 95% of that total amount of isotope incorporated. The cytotoxicity assay was established at multiple effector:target ratios and dose-response curves were generated by plotting percent SCR vs. the number of effector cells to compute lytic units.

Target-cell binding assay. The target-cell binding assay was performed as previously described (20). In brief, pretreated or untreated LGL were incubated with MOLT-4 or K-562 at an effector to target ratio of 1:1. LGL (1×10^6 cells/ml) were incubated with target cells (1×10^6 cells/ml) for 30 min at 4°C. The cells were then resuspended and a minimum of 200 effector cells were counted in a Neubaur counting chamber covered with acridine orange-coated cover slips with simultaneous UV light and phase-contrast illumination to determine the number of effector-target conjugates. The utilization of acridine orange-coated cover slips and UV light permitted clear distinction of effector cells from target cells (18).

Results

Activation of alloantigen-specific memory cells by anti-T3 MoAb. Memory cells, generated in long-term MLCs, were activated by anti-T3 in the absence of the original priming stimulus. Results from seven consecutive experiments are summarized in Fig. 1. Exposure of memory cells to anti-T3 resulted in significant proliferation as determined by [^{3}H]thymidine incorporation (Fig. 1 *A*) and secondary cytolytic activity as determined in the 4-h ^{51}Cr-release assay (Fig. 1 *B*). [^{3}H]Thymidine incorporation was 1,935±259 cpm/culture (mean ±SE from seven experiments) when memory cells were co-cultured with irradiated syngeneic cells (responder cells in the long-term primary MLC) and 11,853±2,131 cpm/culture when memory cells were exposed to 50 ng/ml of anti-T3. This augmentation in [^{3}H]thymidine uptake was significant at $P < 0.01$ by paired t test. As expected, memory cells exhibited significant proliferation when exposed to the original priming stimulus (Fig. 1 *A*).

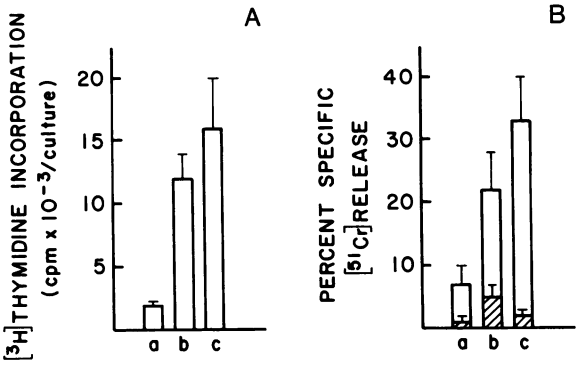

Figure 1. Activation of memory cells with anti-T3. Memory cells (1×10^6 cells/ml) were co-cultured with equal numbers of irradiated syngeneic cells without (*a*) or with anti-T3 (50 ng/ml) (*b*) or with allogeneic cells (*c*). [^{3}H]Thymidine incorporation during 48–64 h of culture is shown in *A* and cytolytic activity found with specific allogeneic PBM (□) and syngeneic PBM (▨) as target cells is shown in *B*. Percent SCR was calculated using the formula $(ER - SR) \div (MR - SR) \times 100$, where *ER* is experimental release, *SR* is spontaneous release and *MR* is maximum release. Results from seven experiments (mean±SE). The effector:target ratio was 50:1 in the ^{51}Cr-release assay.

Activation of memory cells by anti-T3 also resulted in their acquisition of specific secondary cytolytic activity (Fig. 1 *B*). Percent SCR was 7±3% when memory cells co-cultured with irradiated syngeneic cells were effector cells and target cells were PBM from individuals whose cells functioned as stimulator cells in the long-term primary MLC. Percent SCR was 22±6% when memory cells activated with anti-T3 were effector cells. The augmentation in cytolytic activity by anti-T3 reached statistical significance at $P < 0.01$ level by paired t test. As expected, significant augmentation of cytolytic activity ($P < 0.01$) was also found when memory cells that were co-cultured with irradiated allogeneic cells (original priming stimulus) were used as effector cells (Fig. 1 *B*).

The secondary cytolytic activity found with memory cells activated with the original sensitizing stimulus or with anti-T3 was specific for the original priming stimulus. Significant cytotoxicity was found only when specific allogeneic PBM (original sensitizing stimulus) were target cells and no significant cytotoxicity was found when nonspecific allogeneic PBM (HLA-A, B antigens different from specific allogeneic PBM) were target cells. In six experiments, the augmentation in cytolytic activity resulting from anti-T_3 pretreatment or from the exposure of memory cell to specific alloantigen stimulus reached statistical significance at $P < 0.01$ level only when specific allogeneic PBM were target cells (Fig. 2 *A*). No such augmentation in cytolytic activity was found when nonspecific allogeneic PBM were target cells (Fig. 2 *B*). Additional proof for the lack of induction of nonspecific cytolytic activity by anti-T_3 was provided by the finding of lack of significant cytolytic activity when PBM, rather than memory cells, were activated

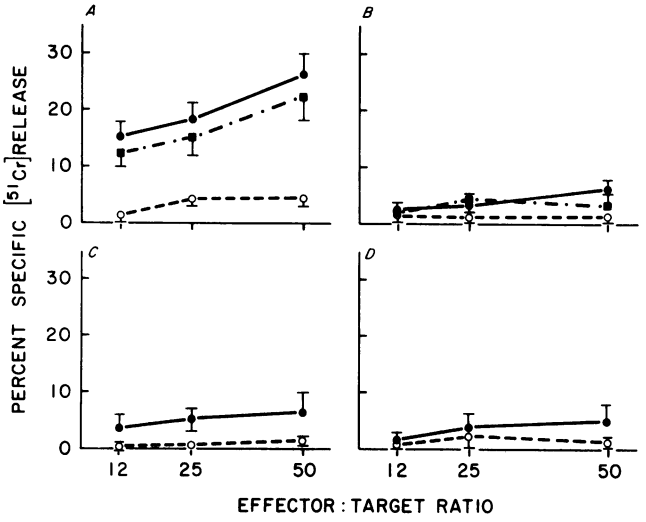

Figure 2. Induction of specific secondary cytotoxic activity by anti-T3. Memory cells (1×10^6 cells/ml) were incubated with equal number of syngeneic PBM without (– – o – –) or with 50 ng/ml of anti-T3 (— ● —) or with specific allogeneic PBM (—· ■ ·—) for 3 d at 37°C. At the end of incubation, the cells were retrieved, washed twice, and used as effector cells at multiple effector:target ratios shown. Results (mean±SE) found in six experiments with ^{51}Cr-labeled specific allogeneic PBM as target cells is shown in *A* and with nonspecific allogeneic PBM as target cells in *B*. In *C* and *D*, results found in three experiments where effector cells were PBM incubated with 50 ng/ml of anti-T3 (— ● —), or without anti-T3 (– – o – –) for 3 d at 37°C and target cells were allogeneic PBM (*C*) or syngeneic PBM (*D*) are shown.

with anti-T_3 and used as effector cells in the ^{51}Cr-release assay in which allogeneic or syngeneic PBM were target cells (Fig. 2, *C* and *D*).

Memory cells activated with the original sensitizing stimulus or with anti-T3, however, exhibited significant cytotoxic activity when MOLT-4 were used as target cells. Percent SCR in six experiments was 6±4% with memory cells co-cultured with syngeneic PBM as effector cells and percent SCR was 39±4% with memory cells activated with the original priming stimulus as effector cells. Percent SCR was 45±3% with anti-T3-activated memory cells.

The anti-T3-associated induction of specific secondary cytolytic activity was independent of new DNA synthesis. Exposure of irradiated memory cells to anti-T3 resulted in significant augmentation of cytolytic activity. In three experiments, percent SCR increased from 3±2 to 18±4% when memory cells irradiated prior to activation with anti-T3 were effector cells and specific allogeneic PBM were target cells.

The expression and the role of T3 antigen on memory cells activated with anti-T3 was examined since anti-T3 is known to modulate T3 antigen (1) and the antigen itself appears to be critical for the expression of cytolytic activity (5, 6). Phenotypic characterization of memory cells with monoclonal antibodies directed at T cell-associated antigens revealed the presence of T3 antigen, albeit at lower level, after activation of memory cells with anti-T3. 90 to 95% of all cells expressed T11 antigen before and after activation with anti-T3, while 86±5% cells expressed T3 antigen prior to activation with the anti-T3 and 68±6% expressed T3 antigen after activation. The presence of T3 antigen appeared to be important for the expression of specific secondary cytolytic activity, but not for NK cell-like activity exhibited by memory cells activated with anti-T3. In five experiments, addition of 0.5 μg/ml of anti-T3 to the cytotoxicity assay resulted in 39±8% inhibition when anti-T3 activated memory cells were effector cells and specific allogeneic PBM were target cells. In the same experiments only 5±8% inhibition of cytotoxicity was found when anti-T3 activated memory cells were effector cells and MOLT-4 were target cells.

Monoclonal antibodies directed at T4 or T8 antigen failed to activate memory cells. No significant proliferation or induction of cytolytic activity was found when memory cells were exposed to anti-T4 or anti-T8 under identical experimental conditions (data not shown).

Augmentation of natural killer cell activity by anti-T3 MoAb. LGL exhibited significant augmentation of NK cell activity when exposed to 10 ng/ml of anti-T3. Results from seven experiments with MOLT-4 as target cells are shown in Fig. 3 *A*, and in four experiments with K-562 as target cells in Fig. 3 *B*. Pretreatment of LGL with anti-T3 resulted in significant augmentation of NK cell activity at every effector:target ratio tested with maximum augmentation at the lowest effector:target (*E/T*) ratio. Percent SCR with MOLT-4 as target cells increased from 41±7% to 77±7% (50:1 *E/T* ratio), from 30±5% to 65±8% (25:1 *E/T* ratio) and from 17±4% to 53±8% (12:1 *E/T* ratio) when anti-T_3 pretreated LGL were effector cells. This augmentation of NK cell activity by anti-T_3 pretreatment was significant at the $P < 0.005$ level at every effector:target ratio tested (Fig. 3 *A*). Similar augmentation of NK cell activity was also found in four experiments when K-562 cells were target cells. Percent SCR increased from 36±9% to 65±7% (50:1 *E/T* ratio, $P < 0.05$), from 24±9% to 52±6% (25:1 *E/T* ratio, $P < 0.025$) from 16±7% to 42±6% (12:1 *E/T* ratio, $P < 0.005$) with pretreatment of LGL with anti-T3.

The increased NK cell activity was not secondary to increased binding of target cells by LGL pretreated with anti-T3. In the target cell-binding assay, 35±1% of untreated or pretreated LGL formed conjugates with MOLT-4 cells in four experiments (Fig. 3 *C*) and 37±3% of untreated and 44±4% of anti-T3 pretreated LGL formed conjugates with K-562 target cells (Fig. 3 *D*).

The monoclonal antibodies directed at T4 or T8 antigen did not affect NK cell activity. Results using MOLT-4 as target cells are shown in Fig. 3 *E*. As expected, pretreatment of LGL with anti-T3 resulted in significant augmentation of NK cell

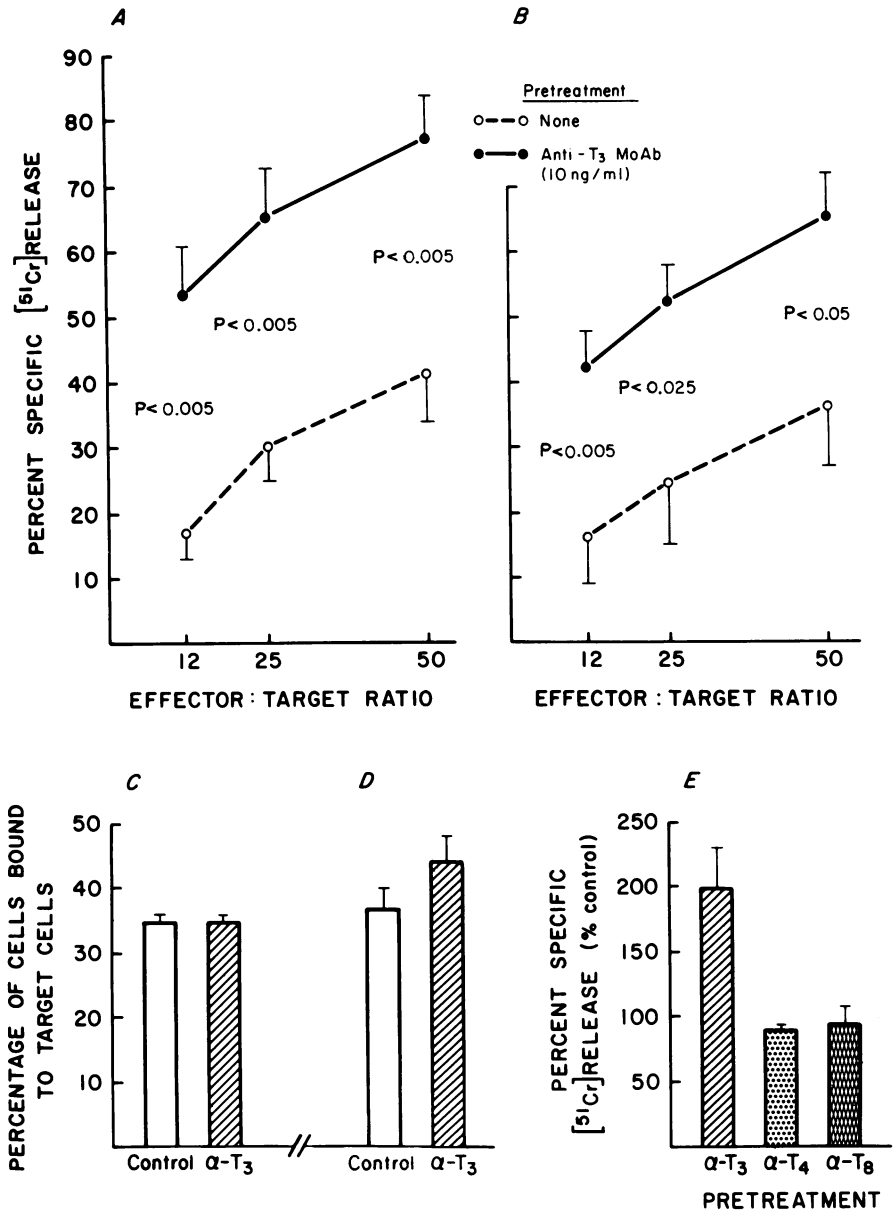

Figure 3. Augmentation of NK-cell activity by anti-T3. LGL were exposed to 10 ng/ml of anti-T3 for 16 h at 37°C in a 5% CO_2:95% air-humidified atmosphere, washed twice, and used as effector cells with MOLT-4 as target cells (n = 7) (*A*) and K-562 as target cells (n = 4) (*B*). The cytotoxicity assay was established at multiple effector:target ratios shown and percent SCR (mean±SE) with each target is shown. The target-cell binding assay was performed at an effector:target ratio of 1:1 and the percentage of effector cells bound to MOLT-4 is shown in *C* and to K-562 in *D*. A minimum of 200 effector cells was counted and the mean±SE from four experiments is shown. In *E*, results found when LGL pretreated with 10 ng/ml of anti-T3 or anti-T4 or anti-T8 were used as effector cells and MOLT-4 were target cells is shown (mean±SE from three experiments). *P* value was calculated by paired *t* test.

activity but percent SCR was 89% and 93% of control when anti-T4 or anti-T8 pretreated LGL were effector cells, respectively. The augmentation in NK cell activity exhibited by LGL pretreated with anti-T3 was not blocked by the addition of anti-T_3 (50 ng/ml–1000 ng/ml) to the cytotoxicity assay. In three consecutive experiments, with MOLT-4 as target cells, percent SCR increased from 39±5 to 60±10% with anti-T_3 pretreatment. In the same experiments, percent SCR was 57±8% when pretreated LGL were effectors and 1 μg/ml of anti-T_3 was added prior to the addition of target cells to the assay.

Specificity of anti-T3-induced augmentation of NK cell activity. Pretreatment of LGL with anti-T3 specifically augmented NK cell activity and did not induce nonspecific cytotoxicity to either allogeneic or syngeneic PBM. Results from three experiments where anti-T3 (10 ng/ml) pretreated LGL were effector cells and MOLT-4 or K-562 or syngeneic or allogeneic PBM were target cells are summarized in Table I. Percent SCR increased from 34±5 to 56±4% with anti-T_3 pretreatment when MOLT-4 were target cells; percent SCR increased from 29±3 to 58±5% with anti-T3 pretreatment when K-562 were target cells. In contrast, percent SCR was

Table I. Specificity of Anti-T3-Induced Augmentation of NK Cell Activity

Effector cell*	Target cell			
Pretreatment	MOLT 4	K-562	Syngeneic PBM	Allogeneic PBM
	% specific ^{51}Cr-release	% specific ^{51}Cr-release	% specific ^{51}Cr-release	% specific ^{51}Cr-release
None	34±5	29±3	1±1	2±1
Anti-T3 (10 ng/ml)	56±4	58±5	3±1	2±2

* LGL were incubated with 10 ng/ml of anti-T3 or without anti-T3 for 16 h at 37°C in a 5% CO_2:95% air humidified atmosphere. At the end of the incubation, the cells were retrieved, washed twice, and used as effector cells at an effector to target ratio of 50:1. Target cells were ^{51}Cr-labeled MOLT-4 or K-562 or syngeneic PBM or allogeneic PBM. Results (mean±SE) from three experiments.

1±1 and 3±1% when untreated or pretreated LGL were effector cells, respectively, and syngeneic PBM were target cells and percent SCR 2±1% without pretreatment and 2±2% with anti-T3 pretreatment when allogeneic PBM were target cells.

Dose-response characteristics of anti-T3-induced augmentation of NK cell activity. Dose-response characteristics of anti-T3 revealed that 1 ng/ml of anti-T3 was sufficient to augment NK cell activity and maximal augmentation was accomplished with 10 ng/ml of anti-T3. While the extent of augmentation of NK cell activity by 1 or 10 or 50 ng/ml of anti-T3 was not different at high effector:target ratios; differences were found at the lowest effector:target ratio tested. As shown in Fig. 4, percent SCR in three experiments with MOLT-4 as target cells was 46±8% (12:1 *E/T* ratio) with LGL pretreated with 1 ng/ml of anti-T3 and percent SCR was 74±8 and 78±6% with 10 and 50 ng/ml of anti-T_3 pretreatment (Fig. 4 *A*). Utilization of lytic units to express NK cell activity augmented by anti-T3 also revealed higher levels of augmentation with 10 or 50 ng/ml of anti-T3 as compared with 1 ng/ml of anti-T3 (Fig. 4 *B*).

Cellular basis for the augmentation of NK cell activity by anti-T3. The augmentation of NK cell activity resulting from anti-T3 pretreatment was dependent upon the presence of T cells in LGL-enriched fractions of PBM. Anti-T3 failed to increase the NK cell activity of LGL depleted of sheep erythrocyte-rosette forming cells or T_3 antigen positive cells. In four experiments, percent SCR was 48±7 (50:1, *E/T* ratio), 44±6 (25:1, *E/T* ratio), and 34±6% (12:1, *E/T* ratio) when effector cells were untreated, LGL-depleted of sheep erythrocyte-rosette forming cells and percent SCR was 48±6 (50:1, *E/T* ratio), 42±5% (25:1, *E/T* ratio), and 38±5% (12:1 *E/T* ratio), when effector cells were LGL depleted of sheep erythrocyte-rosette-forming cells before anti-T3 pretreatment (Fig. 5 *A*). Similar lack of augmentation of NK cell activity was also found when LGL depleted of T3 antigen positive cells were pretreated with anti-T3 after depletion of T3 antigen positive

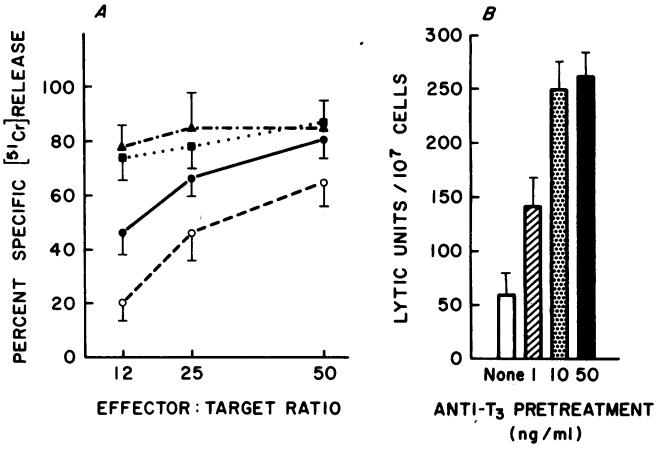

Figure 4. Dose-response characteristics of anti-T3-induced augmentation of NK cell activity. LGL were incubated with or without various concentrations of anti-T3 for 16 h at 37°C in a 5% CO_2:95% air-humidified atmosphere. At the end of incubation, the cells were retrieved, washed twice, and used as effector cells. Percent SCR found with anti-T3 pretreated LGL are shown in *A*. (Anti-T3 concentration: none, — o —; 1 ng/ml, — • —; 10 ng/ml, · · · ■ · · ·; 50 ng/ml, —·— ▲ —·—.) In *B*, NK cell activity is quantitated by calculating lytic units (1 lytic unit = 50% lysis of 5,000 target cells). Results (mean±SE) from three experiments.

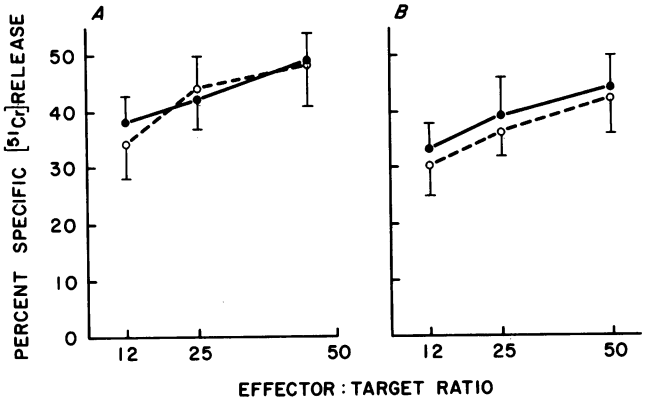

Figure 5. Lack of augmentation of NK cell activity by anti-T3 pretreatment of LGL depleted of sheep erythrocyte rosette-forming cells or T3 antigen-positive (T3+) cells. Nonadherent fractions of PBM were depleted of sheep erythrocyte rosette-forming cells or T3+ cells and subjected to Percoll density gradient centrifugation to obtain LGL-enriched fractions. Results (mean±SE) found in four experiments where LGL depleted of sheep erythrocyte rosette-forming cells were effector cells are shown in *A* and in three experiments where LGL depleted of T3+ cells were effector cells are shown in *B*. The LGL were incubated with 10 ng/ml of anti-T3 (— • —) or without anti-T3 (- - o - -) for 16 h at 37°C, washed twice at the end of incubation, and used as effector cells. The target cells were ^{51}Cr-labeled MOLT-4 cells and the ^{51}Cr-release assay was established at multiple effector:target ratios shown.

cells. In three experiments, percent SCR was 42±6 (50:1, *E/T* ratio), 36±4 (25:1, *E/T* ratio), and 30±5% (12:1, *E/T* ratio) when effector cells were untreated LGL-depleted of T3+ cells, and percent SCR was 44±6 (50:1, *E/T* ratio), 39±7 (25:1, *E/T* ratio), and 33±5% (12:1, *E/T* ratio) when effector cells were LGL depleted of T3 antigen-positive cells prior to anti-T3 pretreatment (Fig. 5 *B*).

Augmentation of NK cell activity found with PBM from immunodeficient patients. PBM were obtained from eight immunodeficient patients. Four of eight patients fulfilled the criteria for the diagnosis of AIDS and the other four patients had received renal grafts at our center and were undergoing immunosuppressive therapy consisting of azathioprine and prednisone. Immune evaluation of acquired immunodeficiency syndrome (AIDS) patients revealed marked suppression of concanavalin A (Con A)- or phytohemagglutinin (PHA)-induced proliferation (10–20% of normal control values) and NK cell activity (20–30% of normal controls). The renal graft recipients also exhibited modest levels of immunodeficiency. Con A- or PHA-induced proliferation and NK cell activity were ~40–50% of normal control values. Pretreatment of PBM obtained from these eight patients with anti-T3 resulted in significant augmentation of NK cell activity. Results found with four patients with AIDS is summarized in Fig. 6 *A*, and with four patients with renal allografts in *B*. Percent SCR increased from 7±2 to 16±2% when anti-T3 pretreated PBM from AIDS patients were effector cells and MOLT-4 were target cells ($P < 0.005$). Percent SCR increased from 15±3 to 49±10% when pretreated PBM from renal allograft recipients were effector cells and MOLT-4 were target cells ($P < 0.025$).

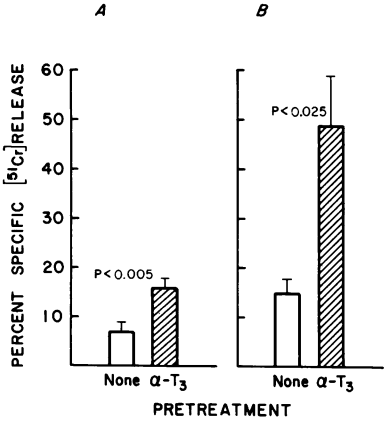

Figure 6. Augmentation of NK cell activity by anti-T3 pretreatment of PBM from immunodeficient individuals. PBM obtained from four patients with AIDS and from four renal graft recipients were exposed to 10 ng/ml of anti-T3 for 16 h at 37°C, washed twice, and used as effector cells. Results (mean±SE) found with MOLT-4 as target cells and PBM from AIDS patients as effector cells are shown in *A* and results found with PBM from renal graft recipients as effector cells are shown in *B*. The ^{51}Cr-release assay was established as 25:1 effector:target ratio and the *P* value was derived by the paired *t* test.

Similar augmentation of NK cell activity was found when anti-T3 pretreated PBM were effector cells and K-562 were target cells (data not shown).

Discussion

The new finding that has emerged from the current investigation is that the monoclonal antibody directed at the T3 molecule is a potent inducer of cytolytic activity. Exposure of antigen-specific memory cells to anti-T3 resulted in their acquisition of specific secondary cytolytic activity and NK cell-like activity. Furthermore, NK cell activity exhibited by LGL obtained from normal subjects and NK cell activity mediated by PBM obtained from patients with AIDS or renal graft recipients were also significantly augmented by anti-T3.

The precise mechanisms involved whereby anti-T3 elicits specific secondary cytolytic activity are unknown. Anti-T3 does share several properties with other mitogens such as PHA, Con A, or oxidizing mitogens, all of which can elicit specific secondary cytolytic activity (11, 21–24). Since anti-T3 can activate T cells (7) and the T3 molecule appears to be closely linked to the T cell antigen recognition complex (1), it is possible that anti-T3 provides the necessary stimulus for triggering the quiescent memory cells into secondary CTL by interaction with the T3 molecule on the surface of memory cells. Alternatively, the differentiation of memory cells into secondary CTL might be secondary to activation of T cell subsets with helper function. The helper cells would then provide the necessary triggering signal. A lymphokine such as IL-2 produced by helper cells is certainly a viable candidate to serve as the triggering signal for the memory cells. The recent findings of Lefrancois et al. (25) that highly purified IL-2 activated murine alloimmune memory cells to exhibit cytotoxicity and our earlier findings that lectin-free soluble factors containing IL-2 activity can effectively substitute for the alloantigen stimulus and activate human alloimmune memory cells (26) lend credence to the above hypothesis that the elicitation of secondary cytolytic activity by anti-T3 might be secondary to its ability to induce IL-2 production. Indeed, the protocols we used to activate memory cells with anti-T3 were associated with IL-2 production.

The specificity characteristics of cytolytic activity resulting from anti-T3-induced activation of memory cells was strikingly similar to the spectrum of cytolytic activity elicited by the original priming stimulus in that both specific secondary cytolytic activity and NK cell-like activity were generated by both stimuli. The specificity of secondary cytolytic activity elicited by anti-T3 or by the alloantigen stimulus was evident when specific and nonspecific allogeneic PBM were used as target cells. Anti-T3-induced cytotoxicity was detected only when specific allogeneic PBM were target cells and no significant cytotoxicity was found when nonspecific allogeneic PBM (HLA-A, B antigens disparate from the original priming stimulus) were used as target cells (Figs. 2, *A* and *B*). In addition, no significant cytotoxicity was found when PBM rather than

memory cells were activated with anti-T3 and used as effector cells and syngeneic or allogeneic PBM were used as target cells (Figs. 2, *C* and *D*). Memory cells activated with anti-T3 or with the alloantigen stimulus, however, exhibited NK cell-like activity. While it is not known whether the secondary cytolytic activity and NK cell-like activity is mediated by the same effector cells as recently proposed by Moretta et al. (27), or by phenotypically different cells as proposed by Zarling and Kung (28), it is of interest that only secondary cytolytic activity exhibited by anti-T3-activated cells was inhibited by the addition of anti-T3 to the cytotoxicity assay and that NK cell-like activity was resistant to such addition. In this context, it is noteworthy that Platsoucas et al. (6), and more recently Moretta et al. (27), found similar differential effects, namely inhibition of specific cell mediated cytotoxicity and lack of inhibition of NK cell-like activity, when monoclonal antibodies (anti-T3, B9-4) directed at T cells were added to the cytotoxicity assay in which the alloantigen-primed cells were used as effector cells and specific allogeneic cells or NK-sensitive target cells were used as target cells.

The differential effects of anti-T3, namely inhibition of primary CTL generation (4) and induction of secondary CTL (Fig. 1), can be explained by the fact that clonal expansion occurring during the generation of memory cells renders anti-T3 to be operationally monospecific when used to activate memory cells, whereas, in primary MLC, activation with a polyclonal activator such as anti-T3 can result in net dilution of antigen-specific clones. Indeed, we and others, have previously reported that several polyclonal activators, including the oxidizing mitogens, activate memory cells to become specific secondary CTL (11, 21–24) while inhibiting the generation of CTL in primary MLCs (29, 30).

The findings that the augmentation of NK cell activity by anti-T3 was dependent upon the presence of T cells in LGL-enriched fractions suggest that the cellular basis for the augmentation of NK cell activity by anti-T3 is secondary to the induction of T cells associated with lymphokine production by anti-T3. Support for this concept comes from several lines of evidence. First, anti-T3 failed to augment NK cell activity of LGL depleted of T cells (Fig. 5, *A* and *B*). Second, a direct effect of anti-T3 on LGL is unlikely in view of the reported absence of T3 molecules on cells capable of mediating NK cell activity (31, 32). Third, anti-T3 is a potent inducer of IL-2 (10) and gamma interferon production (8, 9), both of which can augment NK cell activity (12, 13). Fourth, in preliminary experiments, we have detected interferon production when LGL were pretreated with anti-T3.

The alteration in the NK cell activity resulting from anti-T_3 pretreatment was quantitative rather than qualitative in that pretreated LGL exhibited augmented cytotoxicity to NK-sensitive target cells only and not to syngeneic or allogeneic PBM (Table I). Furthermore, as reported by others (6), the NK cell activity was not inhibited by the addition of anti-T3 to the cytotoxicity assay.

The dose-response characteristics, with respect to anti-T3 ability to augment NK cell activity, were quite similar to dose-response curves for anti-T3-induced mitogenesis of PBM reported by Van Wauwe et al. (7) and Chang et al. (5). Furthermore, a plateau effect at higher concentrations of anti-T3, as reported by others (5, 7), was witnessed in our study (Fig. 4, *A* and *B*).

Several lines of evidence support the hypothesis that the augmentation of cytolytic activity resulting from anti-T3 pretreatment is not dependent upon anti-T3 associated proliferation and is independent of new DNA synthesis. The evidence include the following: (*a*) LGL preincubated with anti-T3 for 16 h only exhibit significant augmentation. In fact, pretreatment of LGL with anti-T3 for 4 h is sufficient to augment NK cell activity; (*b*) Irradiated memory cells can be successfully activated with anti-T3 to exhibit specific secondary cytolytic activity; (*c*) Agents such as cyclosporine or methylprednisolone that inhibit anti-T3-associated lymphokine production (IL-2 and gamma interferon) inhibit anti-T3-associated augmentation of NK cell activity and specific secondary cytolytic activity. (Suthanthiran, M. unpublished observations.)

The clinical significance of the findings of this study is twofold. First, the ability of anti-T3 to generate potentially graft destructive alloimmune responses might contribute, at least in part, to the occurrence of re-rejection episodes in renal graft recipients treated with anti-T3 (3). Additional immunosuppressive therapy directed at suppression of IL-2 production might make antirejection therapy with anti-T3 more effective than at the present time. Second, and perhaps more important, the immunopotentiating property of anti-T3, an obviously disadvantageous property from the perspective of organ transplantation, can be beneficially exploited to rectify impaired cytolytic activity found in immunodeficient individuals. This goal can be accomplished without the attendant immunosuppressive effect of anti-T3 by activating cells ex vivo. The use of anti-T3 to activate cells has several advantages over the use of lectins to activate cells, such as rigorous specificity of anti-T3 for T cells (1, 2, 7), potency of the triggering signal (7), and the lack of any significant complications even when anti-T3 is administered intravenously to renal graft recipients (3). Furthermore, the ability of anti-T3 to induce lymphokine production (8–10), which in turn can augment additional cytocidal mechanisms, such as NK cell activity (12, 13) makes this an attractive approach to activate cells to expand the cytotoxic armamentarium of the host. Additional studies examining the immunopotentiating properties of anti-T3 MoAb appear warranted.

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