

EXPERIMENTAL AUTOIMMUNE THYROIDITIS

In Vitro Cytotoxic Effects of T Lymphocytes on Thyroid Monolayers*

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Earlier studies have established a murine model for human chronic lymphocytic thyroiditis by demonstrating the effects of injecting mouse thyroglobulin (MTg)¹ with either complete Freund's adjuvant (CFA) (1–3) or lipopolysaccharide (4). The experimental autoimmune thyroiditis (EAT) requires T cells for its induction in high responder mice. The *H-2*-linked responsiveness to MTg is encoded by the *I-A* subregion (5). Moreover, recent investigations (6–9) have provided evidence for the existence of T cells reactive with this self-antigen. Lymph node T cells from immunized high responder mice proliferated in culture upon MTg stimulation in vitro (7, 8). The proliferative response was maximal 8–14 d after immunization. It serves as an early in vitro indicator of subsequent lymphocytic infiltration into the thyroids of high responder mice, because similarly immunized low responder mice displayed little blastogenesis and thyroid damage. Furthermore, thyroglobulin-specific T cells responded to repeated injections of soluble, syngeneic MTg (9); even in the absence of adjuvant, all the animals produced autoantibodies and ~50% had thyroid lesions. Studies by others (10, 11) also showed that lymph node T cells cultured from syngeneic thyroid monolayers became blastogenic. We conclude from these findings that EAT is initiated by autoreactive T cells with receptors for MTg. Because suppressor T cells can also be activated by soluble, native MTg (12), we further suggest that EAT is regulated by the clonal balance of T cell subpopulations (13).

Despite the known immunologic manifestations of the disease in both the mouse and human, the regulatory mechanisms and the interplay of T cell subsets in EAT are difficult to dissect. One prime reason is that the effector mechanisms that culminate in tissue damage are complex. A second reason has been our inability to measure thyroid damage in vitro. We therefore initiated studies in vitro on the effector mechanisms of EAT by establishing functional thyroid monolayers suitable for use as target cells. We report here the first demonstration of effector cells that are cytotoxic for thyroid monolayers and their T cell characteristics.

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¹ Abbreviations used in this paper: BSA, bovine serum albumin; cAMP, dibutyryl adenosine 3',5'-cyclic monophosphate; CFA, complete Freund's adjuvant; EAT, experimental autoimmune thyroiditis; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HBSS, Hanks' balanced salt solution; ¹¹¹In, indium-111; LNC, lymph node cells; MTg, mouse thyroglobulin; TSH, thyroid-stimulating hormone.

Materials and Methods

Animals. Female CBA/J ($H-2^k$) mice were purchased from The Jackson Laboratory, Bar Harbor, ME and immunized when 8–10 wk old. For syngeneic and allogeneic thyroid cultures, 3–5-mo-old male and female CBA and BALB/c ($H-2^d$) mice were used. These CBA and BALB/c mice were inbred in our colonies and originated from The Jackson Laboratory and Cumberlandview Farms, Clinton, TN, respectively.

Immunization. MTg was prepared from frozen mouse thyroids, kindly supplied by Dr. C. David (Mayo Medical School, Rochester, MN), and purified by column chromatography as previously described (14). CBA/J mice were immunized with MTg (or saline) emulsified in CFA (Difco Laboratories, Detroit, MI) at a 1:1 ratio, as detailed earlier (8); a total of 120 μ g MTg in 0.2 ml was given at four sites (both hind footpads and thighs).

Antisera and Complement. Anti-Thy-1.2 (immunoglobulin M [IgM]), anti-Lyt-1.1 (IgG), and anti-Lyt-2.1 (IgG) monoclonal mouse antibodies were purchased from Cedarlane Laboratories, Hornby, Ontario, Canada. Rabbit antiserum to mouse Ig, purchased from Miles Yeda Ltd., Rehovot, Israel, was used as an amplifying serum. Guinea pig complement was obtained from Grand Island Biological Co., Grand Island, NY. Mouse antiserum specific for $H-2K^k$ products was prepared by immunizing (BALB/c \times A.TL) F_1 mice repeatedly with A/J-derived thymocytes; mouse antiserum specific for $H-2D^k$ molecules was prepared by immunizing (BALB/c \times A.AL) F_1 mice repeatedly with BALB.k thymocytes. These antisera were kindly supplied by Dr. K. W. Beisel, formerly of the Department of Immunology and Microbiology, Wayne State University School of Medicine. Antiserum to MTg was raised in rabbits by subcutaneously injecting MTg in CFA three times at 4-wk intervals (2.0, 1.7, and 0.8 mg MTg in 1.0 ml CFA at multiple sites). Final bleedings were obtained 1 mo after the last injection and the sera were pooled on the basis of high titers of precipitins to MTg. The rabbit antiserum to MTg was diluted 1:10, and absorbed with either MTg-coated erythrocytes (4) or CBA spleen cells at 4°C for 30 min, with 1/10 vol of packed cells.

Tissue Culture Media. Unless specified otherwise, tissue culture medium and supplements were purchased from Gibco Laboratories. The basic supplements for RPMI 1640 were 25 mM Hepes, 2 mM glutamine, penicillin (100 U/ml), and streptomycin (100 μ g/ml). For lymphocyte cultures, additional supplements were 50 μ M 2-mercaptoethanol (Eastman Kodak Co., Rochester, NY) and 1% fetal calf serum (FCS). For the cytotoxicity assay, 5% FCS was used with 50 μ M 2-mercaptoethanol. For thyroid cell cultures, additional supplements were 10% FCS, fungizone (2.5 μ g/ml), gentamicin (100 μ g/ml; Schering Corp., Kenilworth, NJ), and 1 μ M NaI. Batches of FCS were heat inactivated at 56°C for 45 min and selected for each procedure.

Thyroid Cell Cultures. Thyroids were processed by a modification of the procedure of Yen and Charreire (10). CBA or BALB/c mice were killed by cervical dislocation and dipped in 50% ethanol. The thyroids were removed aseptically and minced with fine scissors in 5 ml Hanks' balanced salt solution (HBSS) that contained 1.5 mg/ml collagenase (Sigma Chemical Co., St. Louis, MO). After a 15-min incubation period at 37°C, the thyroid suspension was vigorously pipetted; this procedure was repeated two more times with incubation periods of 10 min until a homogeneous suspension of partially disrupted follicles was obtained. Next, the thyroid suspension was diluted to 15 ml with HBSS that contained 20% FCS, and washed three times by centrifugation at 200 g for 10 min with HBSS containing 10% FCS. It was then resuspended in thyroid culture medium and dispensed into flat-bottomed 96-well microplates (100 μ l/well). On the following day, nonadherent cells were removed by washing the plates twice with HBSS; fresh culture medium was added and was renewed every 3rd d. Because the concentration of thyroid cells in the suspension was difficult to quantitate, the number of thyroids used was taken as a measure. In general, on day 5 of culture, a confluent monolayer was obtained with a thyroid suspension prepared from 12 mice per microplate. Confluent growth was not achieved with <10 thyroid donors per plate. To obtain functional thyroid cells, culture medium was supplemented with 1 mM dibutyryl adenosine 3',5'-cyclic monophosphate (cAMP) (Sigma Chemical Co.) and 60 μ U/ml thyroid-stimulating hormone (TSH) (Sigma Chemical Co.). These concentrations are optimal, as presented in the Results section. Thyroid function was measured by comparing MTg concentration in tissue culture supernatants and cell extracts. Proteins from the cells were extracted with a buffer containing 5 mM Tris HCl, 1 mM EDTA, 1% Nonidet P-40 (pH 9.2), and protease inhibitors (0.5 mM phenylmethylsulfonyl

fluoride and 5 mM iodoacetamide). Supernatants were collected at different intervals after culture. The samples were dialyzed in 0.05 M NH_4HCO_3 , lyophilized, and reconstituted with 200 μl phosphate-buffered saline containing 0.5% Tween 20. The amount of MTg was determined by the enzyme-linked immunosorbent assay (ELISA).

Lymphocyte Cultures. On days 12–18 after immunization, lymph node cells (LNC) were prepared from inguinal and popliteal lymph nodes by dispersion through 80-mesh stainless steel screens. After three washings by centrifugation in HBSS, the cells were resuspended in culture medium at a concentration of $2 \times 10^6/\text{ml}$. For stimulation with MTg, doses of 10–50 $\mu\text{g}/\text{ml}$ were tried. Bovine serum albumin (BSA) (fraction V; Miles Laboratories Inc., Elkhart, IN) served as a control antigen. LNC were then incubated at 37°C in a humidified incubator containing 5% CO_2 in air. The cytotoxicity assay was performed on day 5 of culture when LNC viability determined by trypan-blue exclusion was ~75%. To deplete lymphocyte subpopulations, aliquots of MTg-stimulated LNC in HBSS ($1 \times 10^7/\text{ml}$) were incubated with monoclonal antibodies to either Thy-1.2 (1:50), Lyt-1.1 (1:20), or Lyt-2.1 (1:20) for 45 min at 4°C. After one washing, the cells were resuspended to the same concentration and incubated with rabbit antiserum to mouse Ig (1:300) for 45 min at 4°C. After washing, the cells were further incubated with guinea pig complement at nontoxic dilutions ($\leq 1:32$) for 1 h at 37°C. After a final washing, the cells were either recultured for 24 h or tested immediately for cytotoxicity against thyroid cells.

Cytotoxicity Assay. Thyroid monolayers were labeled with indium-111 (^{111}In) by incubation with 50 μl of ^{111}In -oxine (12 $\mu\text{Ci}/\text{ml}$) in HBSS for 10 min at room temperature. ^{111}In -oxine was either prepared from ^{111}In -chloride (Medi-Physics, Inc., South Plainfield, NJ) as described by Frost et al. (15) or obtained directly from Amersham Corp., Arlington Heights, IL. After one washing with HBSS that contained 10% FCS, the thyroid cells were incubated for 30 min at 37°C, which reduced the background level of radioactivity. After two additional washings, the ^{111}In -labeled thyroid cells were used as targets for in vitro-stimulated LNC. First, 0.1 ml/well of cytotoxicity medium was added. LNC cultured with MTg for 5 d in vitro were resuspended at $2 \times 10^6/\text{ml}$ (except where indicated otherwise) in the cytotoxicity medium and 0.1 ml was added to the labeled target cells in each well. Each determination was performed in quadruplicate. The plates were incubated overnight (16 h) at 37°C in an incubator containing 5% CO_2 in air. The supernatants (150 μl) were carefully removed from each well, and radioactivity was counted in a gamma-scintillation counter (Nuclear-Chicago Corp., Des Plaines, IL). Maximal lysis was determined after the addition of 1 N HCl to target cells cultured in the presence of medium only; radioactivity usually ranged from 50 to 75×10^3 cpm. Spontaneous release usually ranged from 8 to 10×10^3 cpm or 12–19% of the maximum value. ^{111}In -release was calculated from the formula: percent specific release = (experimental release – spontaneous release) cpm / (maximal release – spontaneous release) cpm $\times 100$. The statistical significance of the difference between control and experimental groups was calculated with the two-tailed Student's *t* test.

When target cells were pretreated with antisera (mouse antiserum to K^k or D^k , or rabbit antiserum to MTg), appropriate dilutions were added after the first washing following labeling. Incubation was for 1 h at 37°C and was followed by two additional washings. The test was then performed as described above.

MTg Determination. The concentrations of MTg in thyroid culture supernatants and cell extracts were determined by the ELISA developed in our laboratory according to established procedures (16). Both coating antibody (AN-24) and alkaline phosphatase-conjugated antibody (D-3) to MTg were obtained from mouse hybridoma lines that were selected from a panel of 18 clones (17, 18). The two clones produced antibodies to different mouse-specific determinants of MTg and were propagated in ascitic form. Antibodies in the ascitic fluids were purified by ammonium sulfate precipitation. The antibodies and MTg used for the standard curve and the conjugate were ultracentrifuged at 100,000 *g* for 1 h before testing; all samples were centrifuged at 8,000 *g* for 2 min. The sensitivity of the test was 10–20 ng MTg/ml.

Results

Propagation of Functional Thyroid Monolayers. The addition of TSH and/or cAMP to in vitro thyroid cultures has been shown to promote follicular formation and to

activate thyroid cells (19–21). Activation has been measured by iodide uptake (19, 20), the release of 3,5,3'-triiodothyronine and thyroxine (20) and thyroglobulin (21) into the culture medium, and changes in membrane potential (19). We first measured ^{125}I -uptake of mouse thyroid cells in culture after the addition of TSH and observed little influence on the amount of incorporation (data not shown). We next determined MTg production after the addition of varying amounts of cAMP to the medium. Supernatants from five wells of a microplate, which contained cells from approximately half a thyroid gland, were pooled on day 3 of culture. The cells were washed three times with medium and the cell proteins were extracted with buffer. After dialysis and concentration by lyophilization, the amounts of MTg in the samples were determined by ELISA; three to five twofold dilutions were tested for each sample. As shown in Fig. 1, thyroid cells cultured in medium without cAMP contained negligible amounts of MTg. The amount of MTg recovered from the cell extracts increased significantly ($P < 0.001$) when 0.25, 0.50, or 1.0 mM cAMP was added. MTg concentrations that could be measured in the culture supernatants were significantly increased by the addition of 0.50 and 1.0 mM cAMP, compared with 0.25 mM ($P < 0.010$) or no cAMP ($P < 0.001$).

To determine if MTg yields could be further increased, the combined use of cAMP and TSH was compared with that of TSH alone. Because thyroid cells optimally

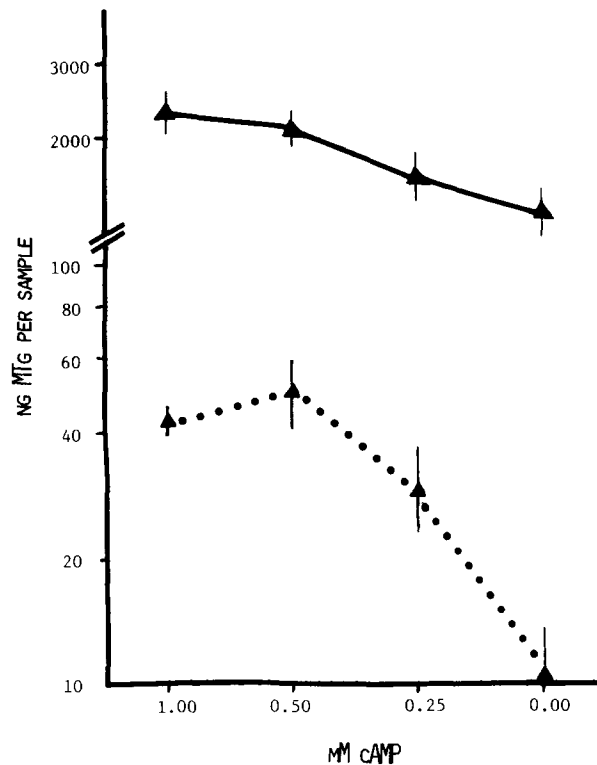


FIG. 1. Amounts of MTg in the supernatant (—) and in the extract of cells (···) from primary CBA thyroid cells cultured at different concentrations of cAMP. MTg concentration was determined on day 3 by comparing extinction values from three to five dilutions per sample (vertical bars indicate standard deviations) with an MTg standard curve in the ELISA.

form monolayers between days 5 and 7, we obtained supernatant samples sequentially on days 3, 6, and 9 of culture. After each sampling, the cells were washed twice before fresh medium was added. Again, five wells were pooled and the amounts of MTg were determined. Fig. 2 indicates that the addition of TSH alone enabled MTg to be produced at a low level up to day 3 (graph A). However, combined with 1 mM cAMP, TSH markedly increased the amount and duration of MTg production (up to 9 d); it was still effective at a dose of 31 μ U/ml. Thus, 60 μ U TSH/ml and 1 mM cAMP were routinely added to propagate thyroid target cells for the cytotoxicity assay. Because the amount of MTg produced gradually decreased during the culture period, we used the target cells no later than 7 d in culture. At this time, the monolayer was confluent and usually contained no more than 20% fibroblasts.

Generation of Effector Cells Cytotoxic for Thyroid Monolayers. Previous studies from our laboratory (7, 8) have shown that LNC from immunized good responder mice proliferate in vitro in the presence of MTg. Thymidine uptake was optimal on day 5 after stimulation with MTg doses of 25–50 μ g/ml (8). Furthermore, the proliferative response appeared to be an early in vitro correlate of lymphocyte infiltration, which is the effector phase of autoimmune thyroiditis. To generate effector cells, we first cultured LNC on day 12 after immunization with 10, 25, or 50 μ g MTg/ml, and measured their cytotoxicity for 111 In-labeled thyroid cells in a 16-h assay on days 4, 5, and 6 of culture. The cytotoxic response was very similar when measured on day 5 or 6 (35 vs. 34% and 63 vs. 75% on two occasions), but none was observed on day 4. Cytotoxicity was usually demonstrable when LNC were cultured with 10 or 25 μ g MTg/ml. We next determined the optimal effector cell concentration with a standard monolayer of thyroid target cells in the cytotoxic reaction. Specific 111 In-release increased in proportion to the number of effector cells added, up to $\sim 3 \times 10^5$ per well (Fig. 3). This titration led us to select a dose of 2×10^5 effector cells in subsequent experiments.

Specificity of the generation of in vitro cytotoxicity for thyroid cells was next investigated; a representative experiment is presented in Table I. No cytotoxicity was observed when LNC from mice immunized with MTg were cultured with 25 or 50 μ g BSA/ml instead of MTg, or when LNC derived from animals immunized with saline and CFA were cultured with 10 or 25 μ g MTg/ml. The strain used for the

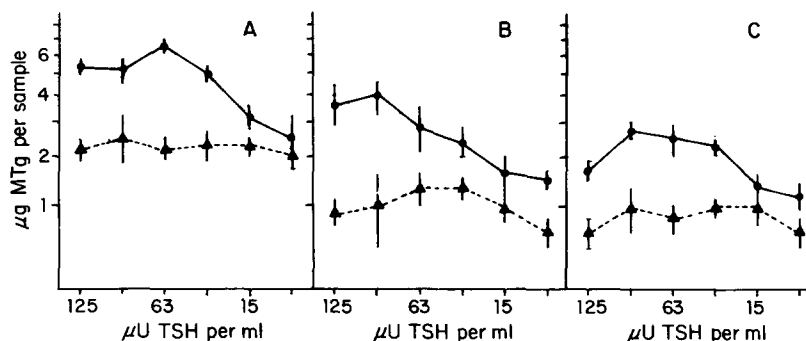


FIG. 2. Amounts of MTg in the supernatant of primary CBA thyroid cell cultures at different concentrations of thyroid-stimulating hormone with (●) or without (▲) 1 mM of cAMP. Samples were obtained on day 3 (A), 6 (B), or 9 (C) of culture. MTg concentrations were determined by means of the ELISA, as indicated in the legend to Fig. 1.

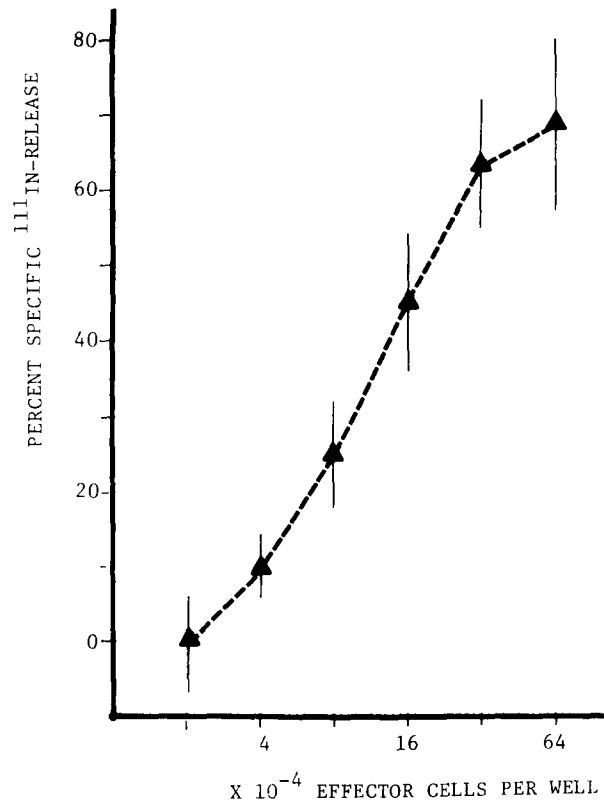


FIG. 3. Relationship between percent specific ¹¹¹In-release from a standard CBA thyroid monolayer and increasing concentrations of effector cells. LNC were obtained from MTg-immunized mice and stimulated in vitro with 10 μ g MTg/ml for 5 d. Vertical bars indicate standard deviations of quadruplicates.

TABLE I
*Cell-mediated Cytotoxicity for Thyroid Cultures by Lymphocytes from Immunized CBA Mice after In Vitro Stimulation with MTg**

Immunization	Antigen added to culture	Percent specific ¹¹¹ In-release \pm SD from thyroid target cells	
		CBA	BALB/c
120 μ g MTg in CFA	10 μ g MTg/ml	34 \pm 9	3 \pm 4
	25 μ g MTg/ml	85 \pm 12	ND \ddagger
	50 μ g MTg/ml	57 \pm 10	3 \pm 8
	25 μ g BSA/ml	9 \pm 5	0 \pm 3
	50 μ g BSA/ml	14 \pm 10	1 \pm 1
Saline in CFA	10 μ g MTg/ml	2 \pm 1	ND
	25 μ g MTg/ml	8 \pm 5	ND

* LNC were collected on day 14 after immunization and were incubated in vitro with MTg or BSA. Cytotoxicity was measured on day 5 of culture.

\ddagger Not determined.

TABLE II
*MTg Specificity of the Cytotoxic Response of Immune CBA LNC**

Series	Antigen added to culture	Thyroid target cell preincubation	Percent specific ¹¹¹ In-release ± SD‡
A	10 µg MTg/ml	Normal rabbit serum	44 ± 10
		Rabbit anti-MTg serum	9 ± 6
B	25 µg MTg/ml	Rabbit anti-MTg serum absorbed with MTg-coated erythrocytes	63 ± 15
		Rabbit anti-MTg serum absorbed with CBA spleen cells	2 ± 4

* LNC were obtained on day 12 after immunization with 120 µg MTg in CFA. Cytotoxicity was performed on day 5 of culture with MTg.

‡ Numbers are averages of two identical experiments with similar values of maximal cytotoxicity. The differences between experimental and control values were statistically significant ($P < 0.010$) within each experiment before the results were pooled.

thyroid target cells was also important: LNC from CBA mice were cytotoxic for labeled target cells from syngeneic, but not allogeneic (BALB/c), thyroids.

The need for MTg in antigen recognition by immune lymphocytes in the generation and expansion of cytotoxic cells also applied to target cell destruction. As shown in Table II, preincubation of ¹¹¹In-labeled thyroid monolayers with rabbit anti-MTg serum (1:100) blocked the cytotoxic reaction (series A). The blocking activity of antibodies to MTg was removed by absorption with MTg-coated erythrocytes, which restored cytotoxicity to 63% (series B). In contrast, blocking activity was retained after absorption with CBA spleen cells, an indication that inhibition of cytotoxicity was not due to the possible presence of antibodies to murine H antigens. These findings show that the cytotoxic reaction depends upon recognition by the effector cells of MTg determinants expressed on the thyroid cell membrane.

Characterization of Cytotoxic Effector Cells. We have shown above that immune CBA LNC stimulated in vitro with MTg became cytotoxic for syngeneic but not allogeneic thyroid target cells. The *H-2* requirement in syngeneic cell killing was then studied by pretreatment of thyroid monolayers with monospecific alloantisera to the *K^k* (1:40) and *D^k* (1:20) regions. Table III shows that incubation with either anti-*K^k* or anti-*D^k* serum resulted in ~50% reduction in cytotoxicity (28–31% ¹¹¹In-release), compared with treatment with normal mouse serum (66% ¹¹¹In-release). When target cells were preincubated with anti-*K^k* and anti-*D^k* sera combined, cytotoxicity was completely abolished.

To determine the surface antigens of the cytotoxic LNC, aliquots of 2×10^7 immune cells that had been cultured with MTg were incubated with monoclonal antibodies to Thy-1.2, Lyt-1.1, or Lyt-2.1, and complement just before the cytotoxic test was performed (day 5 of culture). Rabbit anti-mouse Ig was used as an amplifier. To determine whether cells of different Lyt type played a role, similar treatment with antisera was performed on day 4 of culture, when no cytotoxicity was detectable. The latter cells were subsequently cultured for another day, so that cytotoxicity was measured on day 5. Control LNC (treated with rabbit antiserum to mouse Ig and complement only) were counted, and diluted in cytotoxicity medium to 2×10^5 /well for the cytotoxicity assay. Because all samples contained the same number of LNC

TABLE III
*H-2 Requirement of Cytotoxic Response of LNC from Immunized CBA Mice
 after Stimulation In Vitro by Culture with MTg**

Target cell preincubation	Percent specific ¹¹¹ In-release ± SD‡ from thyroid target cells	
	CBA	BALB/c
Normal mouse serum	66 ± 1	3 ± 4
Mouse anti-H-2K ^k	31 ± 4	
Mouse anti-H-2D ^k	28 ± 2	
Mouse anti-H-2K ^k + anti-H-2D ^k	1 ± 4	

* LNC were obtained on day 14 after immunization with 120 µg MTg in CFA. Cytotoxicity was performed on day 5 of culture with MTg.

‡ Numbers are averages from two identical experiments with similar values for percent cytotoxicity in normal mouse serum controls. The differences between experimental and control values were statistically significant (*P* < 0.010) within each experiment before the results were pooled.

TABLE IV
*Influence of Killing Immune LNC Subsets on Day 4 or 5 after Culture with
 MTg on the Cytotoxic Reaction on Day 5*

Treatment	Percent specific ¹¹¹ In-release ± SD* from thyroid target cells	
	Day 4	Day 5
Anti-Thy-1.2, rabbit anti-mouse Ig, and complement	9 ± 13	9 ± 7
Anti-Lyt-1.1, rabbit anti-mouse Ig, and complement	6 ± 6	57 ± 18
Anti-Lyt-2.1, rabbit anti-mouse Ig, and complement	56 ± 6	4 ± 6
Rabbit anti-mouse Ig and complement	64 ± 17	62 ± 13

* Numbers are averages of three experiments (day 4) or two experiments (day 5) pooled on the basis of statistically significant differences (*P* < 0.010) within each experiment. Experiments were selected on the basis of similar percent cytotoxicity in control cultures (treatment with rabbit anti-mouse Ig and complement only).

before antiserum treatment, the LNC, depleted of subsets, were diluted in volumes identical to the control sample.

Table IV shows that the cytotoxic cells were Thy-1⁺ and Lyt-2⁺. Treatment with Lyt-2.1, but not Lyt-1.1, antibodies just before the cytotoxic assay was performed (day 5 of culture) prevented the cytotoxic response. In contrast, treatment on day 4 with Lyt-2.1 antibody and complement 24 h before the cytotoxic assay was performed did not abolish the cytotoxic response, whereas treatment with anti-Lyt-1.1 antibody and complement did. Thus, despite further incubation in culture after the elimination of Lyt-1-bearing subsets, cytotoxic cells were not generated.

Discussion

The data above show that LNC from MTg-immunized mice became cytotoxic for thyroid monolayers after in vitro culture with MTg and that the effector cells are

Lyt-2-bearing T cells. In the course of these studies, our first concern was to establish functional thyroid cell cultures with a suitable label for assessing target cell destruction. We elected to monitor MTg production as a measure of thyroid function (21) rather than iodide uptake (19, 20) or the release of thyroid hormones (20), reasoning that, as the autoantigen in question, MTg on the thyroid membrane would provide the necessary epitopes for the effector cells. To determine whether MTg found in the supernatant represented newly synthesized protein or the release of preformed protein, cell extracts were also assayed for MTg content. It is apparent that the MTg found in the 3-d supernatant of thyroid cells cultured in the presence of cAMP (≥ 0.50 mM) was at least in part newly synthesized, because at lower concentrations of cAMP, the supernatants contained much less MTg and the amounts recoverable from the cells fell precipitously (Fig. 1). It is not known if, in the absence of cAMP, MTg production ceased early during the 3-d period or never occurred. When varying concentrations of TSH were added in combination with 1 mM cAMP, the amounts of MTg in the medium increased markedly and production continued for 9 d (Fig. 2). When no cAMP was included, varying the concentration of TSH exerted little influence on MTg synthesis. Because confluent monolayers of thyroid culture were observed by days 5–7 and were suitable for use as target cells, they were functional in terms of MTg production at the time of the cytotoxicity assay.

Specificity of the cytotoxic response of LNC for MTg was tested at both the effector and target cell level. Generation and expansion of cytotoxic clones were observed only when MTg was added to the culture medium but not when BSA was used (Table I). LNC from saline-injected mice did not develop cytotoxic cells in culture in the presence of MTg. Cytotoxicity for thyroid targets was blocked by their pretreatment with rabbit antiserum to MTg (Table II). The blocking activity was removed by absorption with MTg-coated erythrocytes but not with CBA spleen cells, which indicates that the blockade was not caused by the possible presence of antibodies to H antigens of the mouse. Thus, the immune LNC recognize MTg in both clonal expansion and target cell destruction.

However, MTg determinants alone on the target cells are insufficient to initiate killing by activated LNC. BALB/c thyroid monolayers labeled with ^{111}In are not destroyed by MTg-stimulated LNC from CBA mice. This *H-2* restriction was verified by finding antisera to *K^k* and *D^k* regions inhibitory to the cytotoxic response (Table III). Each antiserum could reduce ^{111}In -release by $\sim 50\%$, whereas their combined use led to total inhibition. Thus, both *K* and *D* molecules serve as restriction elements in target cell killing. The need for *K* and *D* region recognition resembles the cytotoxic responses of effector T cells to tumor and other modified self-antigens (22) and has been reported in producing lesions in immunized mice given thyroid transplants (23).

Characterization of the cytotoxic cells shows that they and their precursors are Thy-1-bearing T cells. By day 5 of culture, these effector T cells express the Lyt-2 phenotype (Table IV), and the cytotoxic response observed on either day 5 or 6 is similar in magnitude. In contrast, on day 4 of culture, cytotoxicity was not yet measurable. The effector LNC were not removed by killing with Lyt-2 antibodies at this stage, which suggests that most cytotoxic cells were still in their undifferentiated state. Yet when the LNC were exposed to Lyt-1 antibodies on day 4, no further cytotoxic response was observed on day 5. These findings suggest that, although T

cell clones cytotoxic to thyroid monolayers are Lyt-2⁺, they require the presence of Lyt-1⁺ (Lyt-123⁺) cells for their expansion and/or differentiation.

The absence of detectable cytotoxicity in the popliteal and inguinal LNC during the early culture period appears to reflect the *in vivo* state. LNC and spleen cells removed between days 12 and 23 after immunization and tested without prior culture with MTg were not cytotoxic (unpublished observations). The results suggest that sensitized T cells leave these lymph nodes and spleen once they have differentiated to a certain stage, whereas their precursors committed to MTg remain (at least for a period of time). This interpretation accords with our previous report that optimal proliferative response to MTg occurs in popliteal LNC obtained on days 8–14 after immunization, declining by days 21–28 (8). It is also possible that suppressor cells that interfere with responses to MTg develop between 2 and 3 wk after immunization but die during culture, thereby permitting cytotoxic clones to develop *in vitro*. This possibility is under study.

To our knowledge, this study represents the first direct demonstration *in vitro* of a T cell effector mechanism in EAT. Thyroid-specific T cells have been reported to effect EAT in various animal transfer systems (24–26), two of which involve prior *in vitro* culture for 3 d with thyroglobulin (25) or thyroid monolayers (26). Such transfers into normal recipients, which lead to thyroid damage, may involve the recruitment of host cytotoxic clones and/or contain donor cytotoxic precursor cells. Although these studies implicate T cells in EAT, they do not yet explain how damage eventuates. The *in vitro* cytotoxic system described herein permits the dissection of the steps in cellular differentiation and the precise identification of effector cells. Moreover, it may facilitate the study of regulatory mechanisms such as the *D*-end modulation of thyroiditis reported by us earlier (14).

Whether the present observation of cytotoxic mechanisms in EAT is applicable to human thyroiditis remains to be determined. Thyroid membrane molecules other than thyroglobulin may serve as additional autoantigens. As yet, the effector mechanisms that lead to thyroid damage in the human are unclear, despite ample evidence for autoimmune responses. This evidence includes (a) sensitized T cells in the peripheral blood, which can either reduce iodide uptake by cultured thyroid cells (27) or kill antigen-coated mastocytoma cells (28), (b) antigen-specific T cells (29) and plaque-forming cells in the thyroid (30), and (c) circulating antibodies to more than one thyroid antigen. Some of the antibodies have been shown to mediate antibody-dependent cellular cytotoxicity for thyroglobulin-coated erythrocytes (31). For each response, however, it remains difficult to distinguish between cause and effect of thyroid damage. On the basis of our present study, it is clear that direct T cell cytotoxicity may be one prime effector mechanism.

Summary

Effector mechanisms in experimental autoimmune thyroiditis (EAT) were studied *in vitro* by establishing a cytotoxicity system with thyroid target cells. Lymph node cells (LNC) from popliteal and inguinal lymph nodes were obtained from CBA/J mice (8–10 wk old) 12–18 d after immunization with 120 μ g mouse thyroglobulin (MTg) in complete Freund's adjuvant (0.2 ml to both hind footpads and thighs) and were cultured with MTg (10–50 μ g/ml). On day 5 of culture, viable LNC were added to labeled thyroid monolayers and their cytotoxicity was assayed after 16 h. Functional

thyroid target cells, as reflected by MTg production for up to 9 d, were prepared by adding 1 mM dibutyryl adenosine 3',5'-cyclic monophosphate and 60 μ U thyroid-stimulating hormone/ml to the culture medium. On days 5–7, confluent monolayers were labeled with ^{111}In and used as targets. Specific ^{111}In -release ranged from 56 to 85%.

The cytotoxic response is MTg specific and *H-2* restricted. Pretreatment of thyroid target cells with rabbit antiserum to MTg completely inhibited cytotoxicity. Pretreatment with mouse antiserum to either *K^k* or *D^k* products resulted in ~50% inhibition, whereas the combined use of both antisera led to total inhibition. No cytotoxicity was observed when control BALB/c thyroid cultures were the target cells. The kinetics of the expansion of Thy-1⁺ cytotoxic cells by *in vitro* exposure to MTg were then studied. The cytotoxic response required 5 d to develop and was abolished by treating LNC on day 4 with monoclonal antibody to Lyt-1.1, but not to Lyt-2.1, plus complement. In contrast, by day 5, cytotoxicity was abrogated by similar treatment with antiserum to Lyt-2.1, but not to Lyt-1.1. We conclude that cytotoxic cells derived from MTg-immunized mice are Lyt-2-bearing cells but require the presence of Lyt-1-bearing cells for their generation and/or differentiation.

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