

Induction of Severe Granulomatous Experimental Autoimmune Thyroiditis in Mice by Effector Cells Activated in the Presence of Anti-interleukin 2 Receptor Antibody

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Summary

Spleen cells from CBA/J mice immunized with mouse thyroglobulin (MTg) and the adjuvant lipopolysaccharide induce experimental autoimmune thyroiditis (EAT) after transfer to recipient mice if they are first activated in vitro with MTg. EAT induced by cells cultured with MTg is generally moderate in severity and is characterized by a thyroid infiltration consisting primarily of mononuclear cells. Addition of the anti-interleukin 2 receptor (IL-2R) monoclonal antibodies (mAbs) M7/20, 3C7, or 7D4 to spleen cell cultures with MTg resulted in a cell population capable of inducing a more severe type of EAT characterized by extensive follicular destruction, granuloma formation, and the presence of multinucleated giant cells. Recipients of cells cultured with MTg and anti-IL-2R mAb also had higher anti-MTg autoantibody responses than recipients of cells cultured with MTg alone. Activation of cells capable of transferring severe granulomatous EAT and increased anti-MTg autoantibody responses required both MTg and M7/20 in culture and required addition of M7/20 within the first 8 h of the 72-h culture period. CD4⁺ T cells were required for the expression of both the severe granulomatous EAT lesions and the mononuclear cell infiltrates typically observed in murine EAT. The increased anti-MTg autoantibody responses in recipients of cells cultured with MTg and anti-IL-2R mAbs were not restricted to a particular immunoglobulin G (IgG) subclass and included antibody of the IgG1, IgG2A, and IgG2B subclasses. These results suggest that a subset of CD4⁺ T cells capable of inducing severe granulomatous EAT and increased anti-MTg autoantibody responses is preferentially activated when cells are cultured in the presence of anti-IL-2R mAb. Anti-IL-2R mAb may either prevent activation of cells that induce classical lymphocytic EAT or prevent activation of cells that normally function to downregulate EAT effector T cell activity.

Recent studies have established that mAbs directed against surface molecules on T cells or other cells involved in immune responses to autoantigens can prevent the development of many experimentally induced and spontaneous autoimmune diseases (reviewed in references 1-3). Antibodies such as anti-CD4 directed against the CD4 molecule expressed on CD4⁺ T cells and those directed against class II MHC molecules (Ia) expressed on APC have also been shown to be effective in preventing disease when mAb treatment is begun after effector cells have been sensitized or after clinical signs of the disease are evident (4-10). While these observations suggest that anti-CD4 or anti-Ia mAbs might be useful for therapy of human autoimmune diseases, these mAbs also eliminate many cells other than those involved in mediating the autoimmune response. For this reason, it would be desirable to use mAbs that target a more restricted population of cells

to suppress autoimmune disease. Since recently activated T and B cells express IL-2Rs, which are not present on resting cells (11), several studies have recently assessed the ability of anti-IL-2R mAbs to prevent or arrest autoimmune diseases in animals. Anti-IL-2R mAbs have been shown to suppress the development of lupus nephritis in NZB/NZW mice (12), insulinitis in NOD mice (12) and BB rats (13), and thymectomy-induced thyroiditis in Buffalo rats (14). In addition, experimentally induced allergic encephalomyelitis (EAE)¹ and adjuvant arthritis in rats were suppressed by administration of anti-IL-2R mAb to recipients of sensitized cells (15, 16) or by adding

¹ Abbreviations used in this paper: EAE, experimentally induced allergic encephalomyelitis; EAT, experimental autoimmune thyroiditis; MTg, mouse thyroglobulin.

anti-IL-2R mAb to cultures to block the in vitro activation of T cells (17).

Experimental autoimmune thyroiditis (EAT) is a chronic inflammatory autoimmune disease that can be induced in genetically susceptible mice by injection of mouse thyroglobulin (MTg) and adjuvant (18, 19). Splenic lymphocytes from mice primed with MTg and adjuvant can transfer EAT to normal or irradiated syngeneic recipients after in vitro activation with MTg (20). CD4⁺ T cells are required for the induction of EAT in this model (4, 10), and CD4⁺ T cell lines or clones can induce EAT after transfer to irradiated mice (21, 22), suggesting that CD4⁺ T cells are the major effector cells for EAT in mice. Our laboratory has recently used this cell transfer system, together with mAbs directed against various cell surface molecules, to elucidate the cellular mechanisms involved in the development of EAT in mice. These studies established that CD4⁺ T cells and Ia⁺ APC are required for both the induction and expression of EAT (4, 5). Moreover, addition of either anti-CD4 or anti-I-A^k mAb to cultures during in vitro activation of EAT effector cells blocked MTg-specific proliferative responses, and cells cultured in the presence of these mAbs could not transfer EAT to recipient mice (4, 5, 23).

The present studies were initially undertaken in order to determine if activated EAT effector T cells expressed IL-2R. To address this question, we asked whether addition of anti-IL-2R mAb during the in vitro activation of EAT effector cells would block the in vitro activation of EAT effector cells and prevent the ability of cells to transfer EAT. It will be shown that although addition of anti-IL-2R mAb to cultures containing MTg-sensitized cells and MTg effectively blocked the antigen- and mitogen-induced proliferation of MTg-sensitized T cells, cells cultured with anti-IL-2R mAb unexpectedly transferred more severe EAT to mice than did cells cultured with MTg alone. Moreover, recipients of cells cultured with anti-IL-2R mAb produced markedly increased anti-MTg autoantibody responses, and the histopathology of the EAT lesions differed substantially from the typical lymphocytic thyroiditis observed after active immunization with MTg and adjuvant (18, 19) or after transfer of in vitro activated CD4⁺ T cells to normal or irradiated mice (4, 5, 20, 22). Thyroids of mice receiving cells cultured in the presence of anti-IL-2R mAb were almost completely destroyed, and the lesions were primarily granulomatous in nature. Since CD4⁺ T cells are also required for development of this granulomatous form of EAT, these results raise the possibility that a subset of CD4⁺ T cells resistant to the inhibitory effects of anti-IL-2R mAbs has the capacity to induce severe granulomatous EAT in mice. These results may have important significance for furthering our understanding of the regulation of activation of cells mediating different types of autoimmune inflammatory lesions.

Materials and Methods

Mice. CBA/J mice were obtained through C. Reeder at the National Institutes of Health (Bethesda, MD) or were from our breeding colonies at the University of Missouri (Columbia, MO). Male or female mice, 6–8 wk old, were used for all experiments.

mAbs. Culture supernatants containing the mAb M1/87.27.7 (anti-Forssman; rat IgM) (24) were produced from cells obtained from the American Type Culture Collection, Rockville, MD). Culture supernatants containing the mAb M7/20 (anti-IL-2R; rat IgM) (25) were produced from cells supplied by Dr. G. Gaulton, University of Pennsylvania (Philadelphia, PA), and culture supernatants containing the anti-IL-2R mAb 3C7 (rat IgG) (26) and anti-IL-2R mAb 7D4 (rat IgM) (26) were produced from cells supplied by Dr. M. Misfeldt, University of Missouri. The 7D4 and M7/20 culture supernatants inhibited proliferation of the HT-2 cell line induced by rIL-2 (Genzyme, Boston, MA) but had no effect on HT-2 proliferation induced by rIL-4 (kindly supplied by Dr. S. Gillis, Immunex Corp., Seattle, WA). Ascites containing the anti-CD4 mAb GK1.5 were prepared as previously described (4). Mice received 0.3 ml GK1.5 ascites intraperitoneally 4 or 11 d after transfer of MTg-activated spleen cells (4).

Antigen and Immunization of Donor Mice. MTg was prepared as previously described (20). Mice were injected twice intravenously at 10-d intervals with 150 µg MTg and 15 µg LPS (20).

Cell Transfer System. 7 d after the second injection of MTg and LPS, spleen cells from donor mice were prepared at 10⁷/ml in RPMI 1640 containing 25 mM Hepes buffer (MA Bioproducts, Walkersville, MD), 5% FCS (Lot 3061; Hazelton Laboratories, Denver, PA, or Lot 1115774; HyClone Laboratories, Logan, UT), glutamine, MEM vitamin solution, nonessential amino acids, sodium pyruvate, penicillin-streptomycin (all from Hazelton Laboratories), and 5 × 10⁻⁵ M 2-ME. Cells were cultured in 60-mm petri dishes (4 ml/plate) with 20–25 µg/ml MTg at 37°C for 68–72 h (20). Culture supernatants containing M7/20 or other mAbs were added as indicated for each experiment to a final concentration of 5%. Unless indicated otherwise, mAbs were always added at initiation of culture. Cells were harvested, washed twice in balanced salt solution, and 2.5 × 10⁷ cells were transferred intravenously into 600 rad irradiated syngeneic recipients (20, 27). In all experiments, the number of cells transferred to recipient mice was based on the count of the total recovered cells. No correction was made for differences in viability, which, as noted below, was generally lower for cells cultured with mAb M7/20. In some experiments, recipient mice were given 0.25 ml anti-CD4 (GK1.5) ascites 4 or 11 d after cell transfer (4). Recipient thyroids were removed 8, 11, or 19 d after cell transfer. EAT induced by in vitro activated spleen cells is first detectable 7–8 d after cells transfer; the severity of EAT becomes maximal 17–21 d after cell transfer in both irradiated (27) and unirradiated recipients (20).

Evaluation of EAT. Histologic evaluation of thyroid lesions was on a scale of 1+ to 4+ as previously described (20). Briefly, 1+ thyroiditis is defined as an infiltrate of at least 125 cells in one or several foci, 2+ is 10–20 foci of cellular infiltration involving up to one-fourth of the gland, 3+ indicate one-fourth to one-half of the gland is infiltrated, and 4+ indicates that greater than one-half of the gland is infiltrated. The cellular infiltrate in typical EAT lesions consists primarily of small lymphocytes, macrophages, and a few PMN. In the studies reported here, the lesions in many of the recipients of cells cultured with anti-IL-2R mAb also contained large numbers of histiocytes, including multinucleated giant cells, and these lesions were characterized by granulomatous changes (see Results). Thyroids with these latter features were almost totally infiltrated by cells and were also grossly enlarged. Because these thyroids had a more severe form of EAT lesion than found in typical 4+ lymphocytic thyroiditis, the animals with these severe granulomatous lesions are scored as 5+ in the tables.

ELISA. Serum levels of MTg-specific IgG autoantibodies were assessed by ELISA as described in detail previously (27). For some

experiments, the contribution of various IgG subclasses to the total IgG autoantibody response was assessed using developing antisera specific for mouse IgG1, IgG2A, IgG2B, and IgG3 (Southern Biotechnology, Inc., Birmingham, AL) (5).

Proliferation Assay. Spleen cells were plated in 96-well flat-bottomed plates (25860; Corning Glass Works, Corning, NY) at 5×10^5 cells/well in a final volume of 0.2 ml. MTg (25 or 50 $\mu\text{g/ml}$), OVA (50 $\mu\text{g/ml}$), Con A (5 $\mu\text{g/ml}$), PHA (5 $\mu\text{g/ml}$), or LPS (500 $\mu\text{g/ml}$) was added in a volume of 0.1 ml. Plates were incubated for 24, 48, or 72 h, 0.5 μCi [^3H]TdR was added, and cells were harvested 16–20 h later (28). Results are expressed as the mean Δ cpm of triplicate cultures (cpm with antigen or mitogen cpm with media alone). Details of this assay were described previously (28).

Fluorescence Analysis. Spleen cells from MTg/LPS-sensitized donors were cultured in 60-mm plates in media or with culture supernatant containing the mAb M7/20 (5% final concentration) in the presence or absence of 25 $\mu\text{g/ml}$ MTg. After 24, 48, or 72 h, viable cells were isolated on Ficoll-hypaque (Pharmacia Fine Chemicals, Piscataway, NJ). Cells were stained with M7/20, anti-Forsmann IgM, anti-CD4 (GK1.5), or anti-CD8 (53-6.72) (29) followed by a secondary FITC-labeled anti-rat IgG (Southern Biotechnology, Birmingham, AL) or with a FITC-labeled anti-mouse Ig (Southern Biotechnology, Inc.) or anti-Thy-1.2 (Becton Dickinson & Co., Mountain View, CA). After incubation and washing, cells were analyzed by flow cytometry (5).

Results

Cells Cultured with Anti-IL-2R mAb and MTg Transfer Increased EAT and Produce Increased anti-MTg Responses in Recip-

ient Mice. In an initial experiment, spleen cells from MTg/LPS-primed CBA/J mice were cultured for 72 h with MTg in media alone or in media containing the anti-IL-2R mAb M7/20 (Table 1). Cells cultured with M7/20 had no detectable proliferative responses to MTg or to the T cell mitogens Con A (Table 1) or PHA (data not shown), while cells cultured in media alone proliferated in response to MTg, PHA, or Con A. In most experiments, the viability of cells cultured with MTg and M7/20 was slightly lower (50–60% of recovered cells) than that of cells cultured with MTg alone (75–80%), although the percentage of cells recovered (40–50% of input cells) was comparable for both groups. Recipients of 2.5×10^7 cells cultured with MTg alone all had EAT (1–2+) 19 d after cell transfer and all produced anti-MTg autoantibody (Table 1, Exp. 1). Surprisingly, recipients of cells cultured with MTg and the anti-IL-2R mAb M7/20 all developed more severe granulomatous EAT. These mice also had considerably increased anti-MTg antibody responses compared with controls (Table 1, Exp. 1).

Because M7/20 is an IgM antibody, the increased activity of cells cultured with M7/20 might be explained simply by a nonspecific effect of rat IgM or a nonspecific effect of cross-linking IL-2Rs on the cells. This is not the case since MTg-primed spleen cells cultured with M7/20 in the absence of MTg did not transfer EAT or produce anti-MTg in recipient mice (Table 1, Exp. 2), and cells cultured with MTg and normal rat IgM (Table 1, Exp. 3) or anti-Forsmann IgM (data not shown) transferred EAT that was qualitatively and quantitatively similar to that induced by cells cultured with MTg alone.

Table 1. Induction of Severe Granulomatous EAT with Cells Cultured with MTg and Anti-IL-2R mAb

Exp.	Cells cultured with:*	Severity of EAT [†]					Granulomatous lesions [‡]	Proliferation		Anti-MTg IgG [¶]	
		0	1+	2+	3+	4+		5+	MTg	ConA	1/100
								Δ cpm			
1	MTg	0	2	4	0	0	0/6	11,124	80,575	0.274 \pm 0.022	0.085 \pm 0.020
	MTg + M7/20	0	0	0	0	0	6/6	0	0	0.723 \pm 0.019	0.576 \pm 0.018
2	MTg	0	4	2	0	0	0/6	11,434	127,163	0.445 \pm 0.052	0.163 \pm 0.026
	MTg + M7/20	0	0	0	1	3	6/6	102	900	0.862 \pm 0.021	0.764 \pm 0.033
	M7/20 (No MTg)	5	0	0	0	0	NA	ND	ND	0.042 \pm 0.026	0.010 \pm 0.006
3	MTg	0	3	3	0	0	0/6	6,900	36,260	0.278 \pm 0.034	0.104 \pm 0.017
	MTg + Rat IgM	1	5	0	0	0	0/6	9,100	46,540	0.366 \pm 0.031	0.161 \pm 0.034
	MTg + M7/20	0	0	1	0	1	4/5	0	0	0.771 \pm 0.028	0.563 \pm 0.068

* CBA/J donor mice were immunized with MTg and LPS (see Materials and Methods). Primed spleen cells were cultured 72 h with MTg (except in line 3 of exp. 2, where no MTg was added) \pm culture supernatant (5% final concentration) containing the anti-IL-2R mAb M7/20 or 25 $\mu\text{g/ml}$ Rat IgM. Cells were washed and transferred intravenously to 600 rad irradiated CBA/J recipients.

[†] Thyroids were removed 19 d after cell transfer. The numbers of mice with various degrees of severity of EAT are indicated.

[‡] Number of mice/total of group having granulomatous changes in the thyroid.

^{||} [^3H]TdR was added at 72 h and cells were harvested 18 h later. Data are expressed as mean Δ cpm \pm SD of triplicate wells in the presence of MTg (25 $\mu\text{g/ml}$) or Con A (0.5 $\mu\text{g/well}$).

[¶] Sera from recipient mice (19 d after cell transfer) were assessed for MTg-specific IgG autoantibodies by ELISA. Results are expressed as mean \pm SEM of OD₄₁₀ of 1:100 and 1:400 dilutions of serum.

Table 2. Effect of other Anti-IL-2R Antibodies on EAT Induction

Exp.	Cells cultured with:	Severity of EAT					Granulomatous lesions	Proliferation		Anti-MTg IgG	
		0	1+	2+	3+	4+		5+	MTg	Con A	1/100
								Δ cpm			
1	MTg	0	0	5	1	0	0/6	12,420	112,600	0.193 \pm 0.066	0.020 \pm 0.017
	MTg + M7/20	0	0	0	1	3	6/6	0	1,350	0.744 \pm 0.031	0.270 \pm 0.047
	MTg + 3C7	0	0	0	0	4	6/6	10,900	106,640	0.796 \pm 0.042	0.400 \pm 0.069
2*	MTg	0	4	2	0	0	0/6	11,434	127,163	0.445 \pm 0.014	0.163 \pm 0.026
	MTg + 7D4	0	0	1	3	2	0/6	670	0	0.906 \pm 0.045	0.735 \pm 0.087

See footnotes from Table 1. M7/20, 3C7, and 7D4 mAbs were used at a 5% final concentration of culture supernatant.

* Results obtained with the M7/20 mAb in this experiment are shown in Table 1, exp. 2.

Development of increased EAT effector cell activity was dependent on the amount of M7/20 mAb. Cells cultured with MTg and 5% M7/20-containing culture supernatant induced the most severe (5+) granulomatous EAT. Cells cultured with MTg and 1% M7/20-containing supernatant also induced granulomatous EAT that was of 3-4+ severity compared with the 1-2+ mononuclear cell infiltrates in recipients of cells cultured with MTg and 0.2% M7/20 supernatant or with MTg alone (data not shown).

Spleen cells cultured with MTg and other anti-IL-2R mAbs (7D4 and 3C7) also transferred more severe EAT and produced greater anti-MTg autoantibody responses than cells cultured with MTg alone (Table 2). Cells cultured with MTg and 3C7, like those cultured with MTg and M7/20, induced granulomatous EAT in the majority of recipients (Table 2), whereas cells cultured with MTg and 7D4 induced granulomatous EAT in some (data not shown), but not all, experiments (Table 2). The 7D4 mAb, like M7/20, completely suppressed proliferative responses to Con A and MTg, while the 3C7 mAb had relatively little effect on Con A- or MTg-specific proliferative responses. This suggests that induction of cells capable of transferring granulomatous EAT and increased anti-MTg autoantibody are not necessarily dependent on suppression of in vitro T cell proliferation. Moreover, 3C7, like M7/20, prevents binding of IL-2 to the IL-2R (26), but unlike M7/20, 3C7 is an IgG mAb. Like M7/20, 7D4 is an IgM but binds to the IL-2R at a site distal to the IL-2 binding site (26). Therefore, anti-IL-2R mAbs modulate EAT effector cell function in an antigen-specific manner, and these effects are apparently unrelated either to isotype or site of binding to the IL-2R.

Anti-IL-2R mAbs Are Required Early in Culture. To determine when M7/20 mediates its effects during the 72-h culture, MTg/LPS-primed spleen cells were cultured with MTg, and M7/20 was added to separate groups of cultures at 0, 8, 24, or 48 h (Table 3). Addition of M7/20 at 0 or 8 h resulted in augmentation of EAT and anti-MTg antibody by the transferred cells, although in these as well as in other experiments

(data not shown), the most severe lesions and highest antibody responses occurred in recipients of cells cultured with M7/20 at time 0. Addition of M7/20 at 24 or 48 h after culture initiation resulted in little, if any, effect on the ability of cells to induce EAT or to produce anti-MTg, although the proliferative responses of these cells were still markedly reduced when assessed after 72 h (data not shown). These results indicate that M7/20 mediates its effects early in culture and also demonstrates that the effects of M7/20 are not due to carryover of cell-bound M7/20 antibody into the recipient animals.

Histopathology and Kinetics of Development of Granulomatous EAT in Recipients of Cells Cultured with M7/20. The thyroids of recipients of cells cultured with MTg alone appeared grossly unremarkable and contained an inflammatory infiltrate consisting primarily of lymphoid cells and a few scattered neutrophils (Fig. 1, A and B). This infiltrate, which was confined to the thyroid without extension into the adjacent tissue, replaced the thyroid follicles. While the degree of follicle replacement varied with the severity of the infiltrate, even in severely affected (3-4+) glands the follicular cells remained fairly uniform, being low to flat cuboidal cells with a scant eosinophilic cytoplasm (Fig. 1 B).

In contrast, the thyroids of the majority of mice 19 d after receiving cells cultured with MTg and M7/20 differed both quantitatively and qualitatively from those of mice receiving cells cultured with MTg alone. Grossly, the thyroids with extensive (5+) lesions were markedly enlarged, firm, and pale white. Histologically, the thyroids were almost entirely replaced by a diffuse inflammatory infiltrate associated with extensive follicular destruction (Fig. 1 C); only a few residual colloid-containing microfollicles or small follicles were still evident (Fig. 1 D). The infiltrate was characterized by a proliferation of epithelioid histiocytes, scattered multinucleated foreign body-type giant cells (Fig. 1 E), and lymphocytes. Granuloma formation was also present, some containing central necrosis with neutrophilic abscesses (Fig. 1, D and F). Neutrophils, eosinophils, and plasma cells were also evident in

Table 3. *M7/20 Is Required Early in Culture*

Exp.	Cells cultured with:	Severity of EAT						Granulomatous lesions	Anti-MTg IgG	
		0	1+	2+	3+	4+	5+		1/100	1/400
1	MTg	0	2	3	0	0	0	0/5	0.352 ± 0.043	0.146 ± 0.033
	MTg + M7/20 (0 h)	0	0	0	0	0	5	5/5	0.809 ± 0.035	0.633 ± 0.063
	MTg + M7/20 (8 h)	0	0	0	0	4	1	5/5	0.756 ± 0.058	0.465 ± 0.022
	MTg + M7/20 (24 h)	0	0	3	2	0	0	0/5	0.292 ± 0.021	0.076 ± 0.021
2	MTg	2	3	1	0	0	0	0/6	0.281 ± 0.056	0.119 ± 0.034
	MTg + M7/20 (0 h)	0	0	0	0	1	5	6/6	0.974 ± 0.065	0.791 ± 0.076
	MTg + M7/20 (8 h)	0	0	0	3	1	2	4/6	0.725 ± 0.069	0.677 ± 0.115
	MTg + M7/20 (48 h)	0	4	2	0	0	0	0/6	0.298 ± 0.030	0.133 ± 0.030

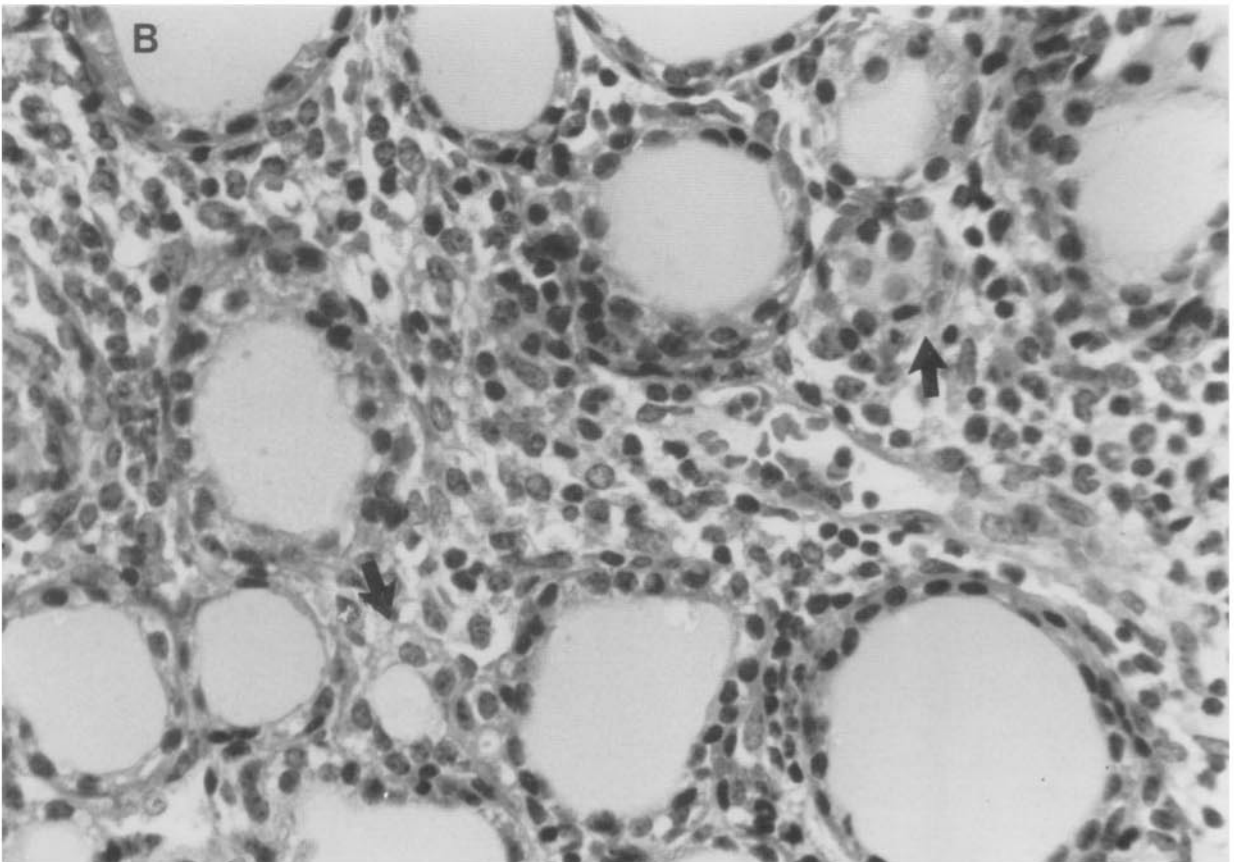
See footnotes from Table 1. M7/20 (5% final concentration of culture supernatant) was added at culture initiation (0 h) or 8, 24, or 48 h after culture initiation.

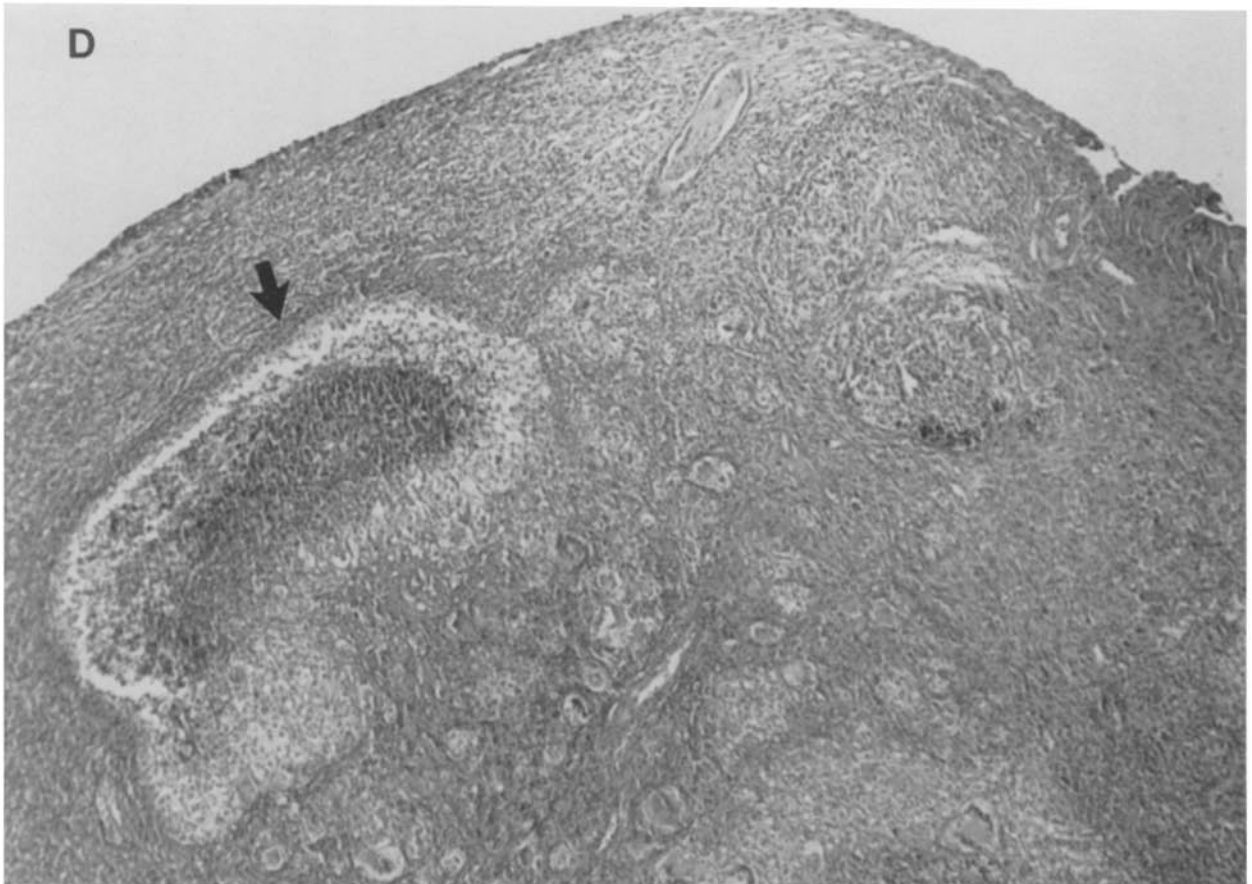
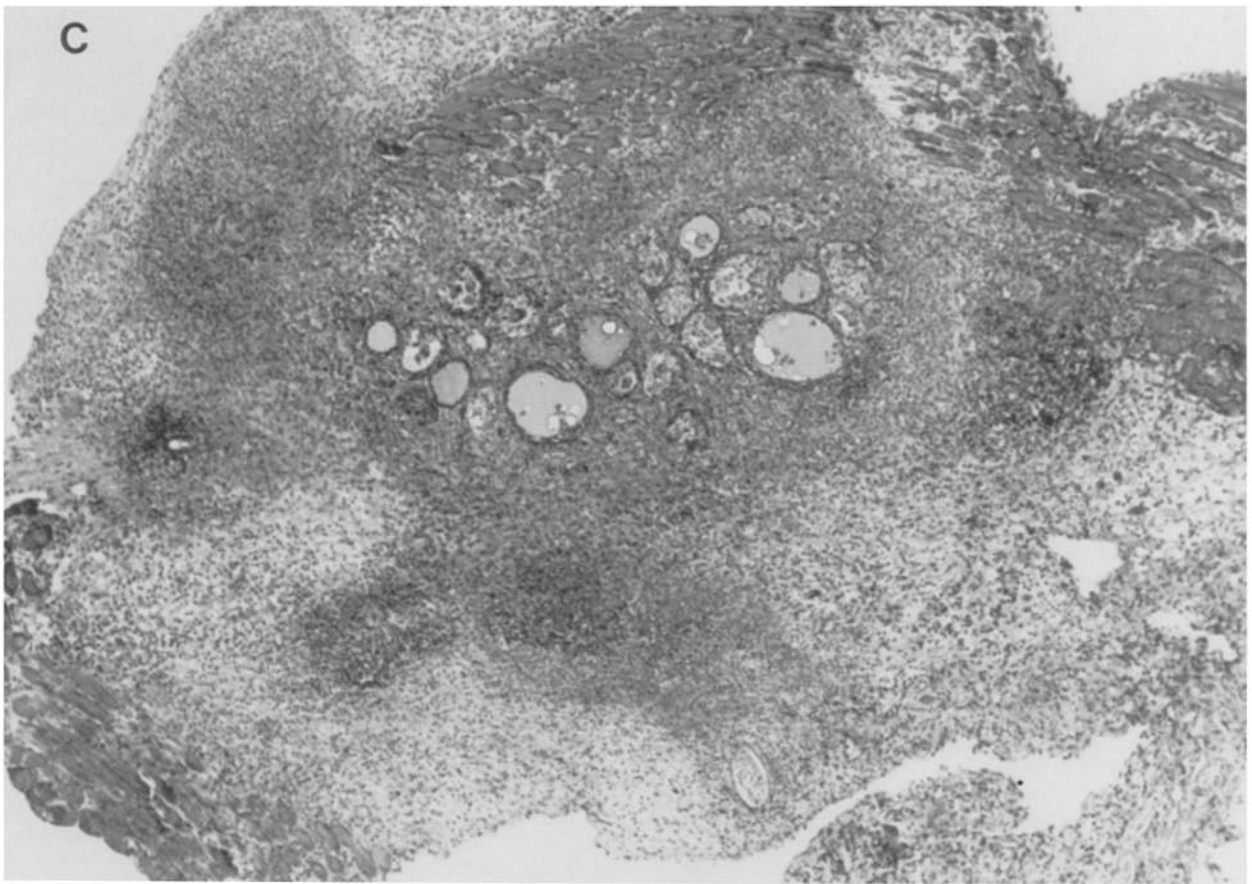
the perifollicular stroma. The degree of acute inflammation varied from a few widely scattered neutrophils to larger focal aggregates in the stroma. Neutrophils also infiltrated the follicles producing luminal microabscesses. The ratio of the acute to granulomatous inflammation varied, but the granulomatous features were always dominant. The follicle cells were also greatly increased in size, many being columnar, with the cytoplasm assuming a pale to granular histiocytoid character. Eventually, these follicle cells crowded out the luminal colloid, forming solid nests. As this point, it was difficult to distinguish the follicular cells from the stromal epithelioid histiocytes (Fig. 1, D-F). In many cases, the inflammatory reaction extended beyond the thyroid to infiltrate the adjacent soft tissue and skeletal muscle (Fig. 1 G). In most cases, the degree of cellular infiltration was much more severe in these animals than in those receiving cells cultured with MTg alone.

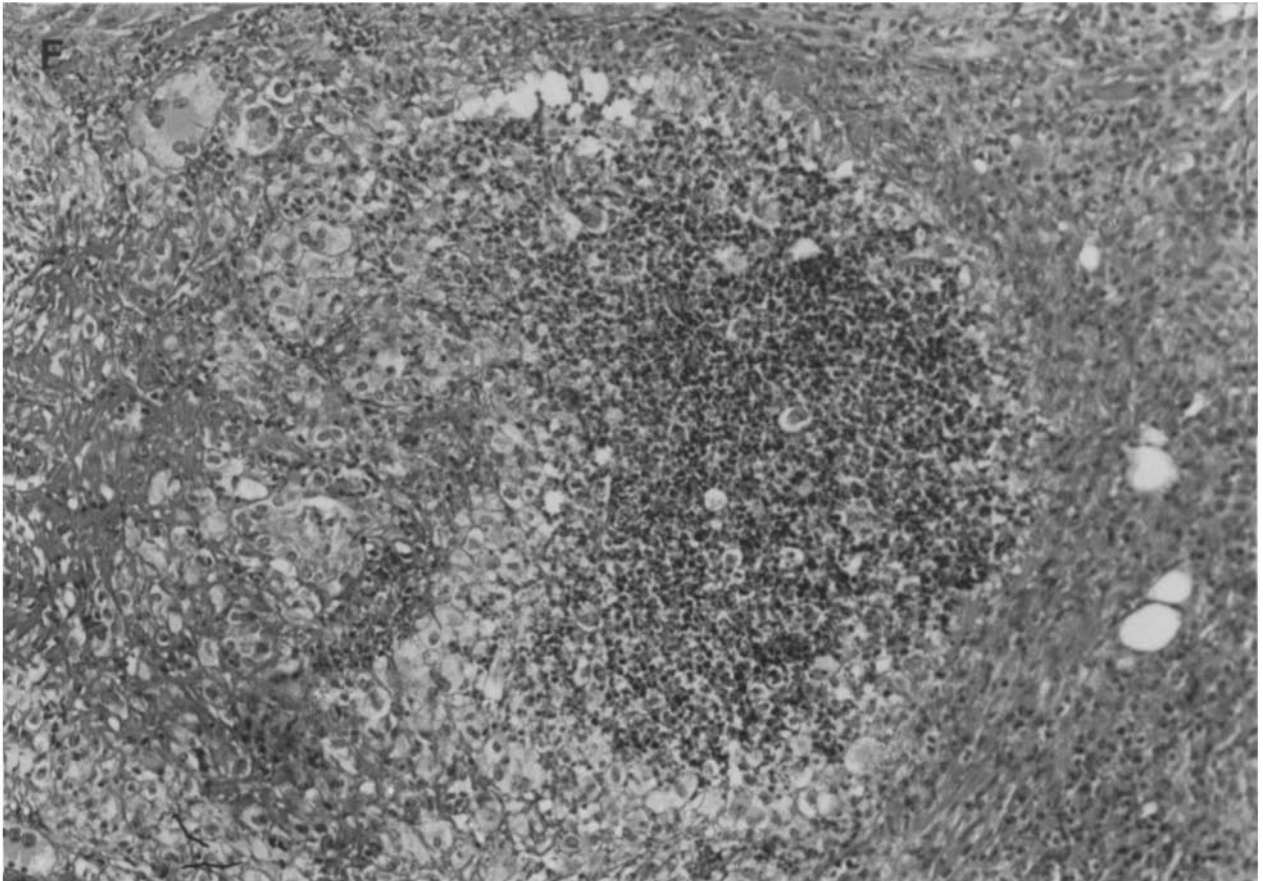
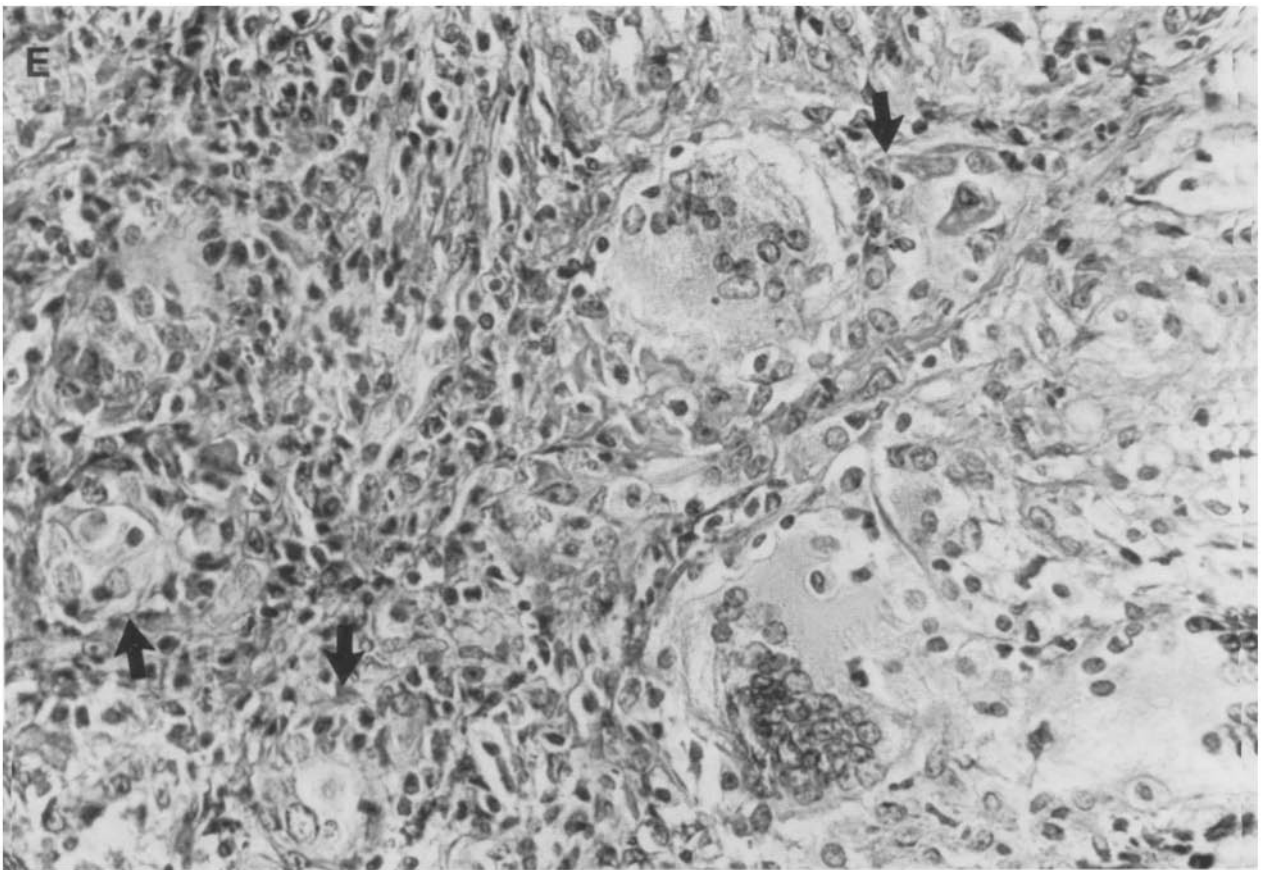
Preliminary kinetics studies were done to begin to examine the time course of development of granulomatous EAT (Table 4). Thyroid lesions in recipients of cells cultured with MTg and M7/20 appeared earlier than in MTg control recipients, with evidence of granulomatous changes by day 8 after cell transfer. The earliest histologic changes consisted of a follicular cell hyperplasia two to three layers of cells in thickness. The cells became more columnar with an increase in cytoplasmic volume, and the nuclei also became enlarged and irregular in shape. This proliferation frequently was more pronounced at one aspect of the follicle, creating an eccentric bulge of the cells outward into the stroma. Here, the cells

simulated the appearance of epithelioid histiocytes and formed multinucleated cells difficult to distinguish in some cases from the stromal histiocytes. The degree of infiltration and granuloma formation increased with time to become maximal at 19 to 21 d after cell transfer. Further studies are in progress to determine more precisely the time course of development of these granulomatous EAT lesions.

CD4⁺ T Cells Are Required for Induction of EAT by Cells Cultured with M7/20. Previous studies from this and other laboratories have established that CD4⁺ T cells are the effector cells for EAT in mice (4, 10, 21, 22). Because the EAT lesions in mice receiving cells cultured with M7/20 are not only more severe but also exhibit histopathologic features not generally observed in control recipients (Fig. 1), it was of interest to determine if CD4⁺ T cells were also required for the development of severe granulomatous EAT. To address this question, recipients of cells cultured with MTg alone or with MTg and M7/20 were given anti-CD4 (GK1.5) mAb either 4 or 11 d after cell transfer (Table 5). As described previously (4), a single injection of anti-CD4 mAb almost completely prevented the development of EAT in recipients of control cells whether the mAb was given before disease development (day 4) or after lesions had begun to develop (day 11). The severity of EAT in recipients of cells cultured with MTg and M7/20 was also markedly reduced by anti-CD4 mAb although the majority of the treated recipients did develop EAT. The thyroids of the anti-CD4-treated recipients of cells cultured with M7/20 contained primarily mononuclear cell infiltrates, although four of the five 2+ le-







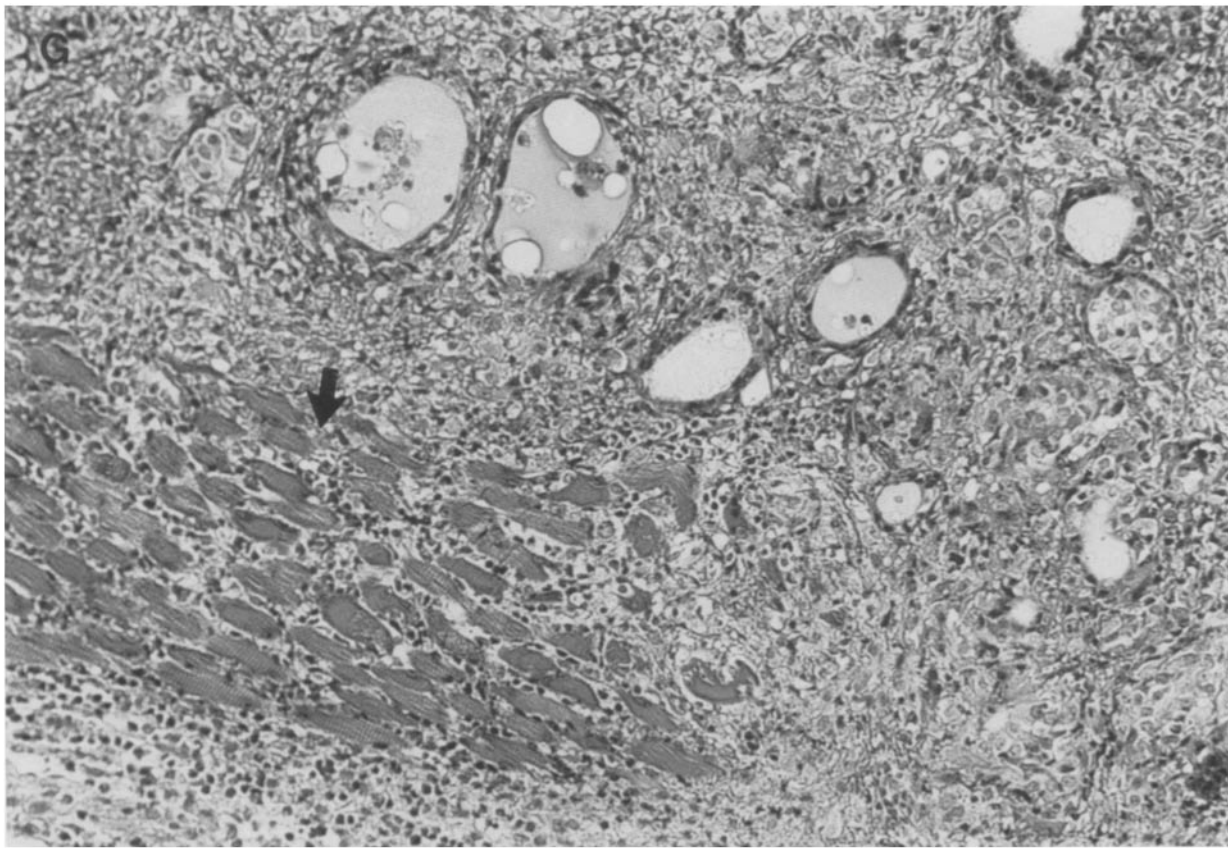


Figure 1. Hematoxylin and eosin (H & E)-stained thyroid sections from CBA/J recipients of cells cultured with MTg (A and B) or MTg + M7/20 (C-G). (A) Conventional experimental lymphocytic thyroiditis inflammatory infiltrate (3+ to 4+) has replaced approximately half of the thyroid parenchyma (H & E; $\times 12$). (B) Higher magnification view of A shows a lymphocytic and mononuclear cell infiltrate between residual thyroid follicles. The follicles are lined by a single layer of cuboidal cells. Some follicles (arrows) are small and atrophic (H & E; $\times 128$). (C) Experimental granulomatous thyroiditis. Low-power view shows diffuse destruction of gland by inflammation that extends into the adjacent soft tissue and muscle (top left and bottom left). A few residual follicles remain. The severity of the inflammation is graded 5+ (H & E; $\times 8$). (D) Experimental granulomatous thyroiditis (5+). The inflammatory infiltrate has replaced the entire gland. Numerous multinucleated giant cells are seen in lower portion of field. A large granuloma (arrow) with central abscess formation is present (H & E; $\times 8$). (E) Granulomatous thyroiditis shows a mixture of large, multinucleated giant cells with neutrophils, and mononuclear histiocytes. A few atrophic follicles (arrows) remain. Note difficulty of distinguishing stromal histiocytes from follicle cells (H & E; $\times 100$). (F) An area of granulomatous thyroiditis shows focal abscess surrounded by a rim of epithelioid histiocytes (H & E; $\times 40$). (G) Lymphohistiocytic infiltrate of granulomatous thyroiditis extends beyond the thyroid into the adjacent skeletal muscle (H & E; $\times 50$).

Table 4. Kinetics of Development of Granulomatous EAT in Recipients of Cells Cultured with MTg and M7/20

Cells cultured with:	Day of EAT assessment*	Severity of EAT						Granulomatous lesions	Anti-MTg IgG [†]	
		0	1+	2+	3+	4+	5+		1/100	1/400
MTg + M7/20	8	0	2	2	1	0	0	3/5	0.710 \pm 0.039	0.337 \pm 0.040
MTg	11	3	2	0	0	0	0	0/5	0.444 \pm 0.088	0.109 \pm 0.031
MTg + M7/20	11	0	0	0	1	4	0	5/5	0.888 \pm 0.053	0.549 \pm 0.086
MTg	14	2	3	0	0	0	0	0/5	0.347 \pm 0.029	0.100 \pm 0.048
MTg + M7/20	14	0	0	0	2	4	0	5/6	0.884 \pm 0.055	0.566 \pm 0.074
MTg	21	0	0	4	1	0	0	0/5	0.420 \pm 0.016	0.066 \pm 0.010
MTg + M7/20	21	0	0	0	1	0	4	5/5	0.957 \pm 0.083	0.776 \pm 0.028

See footnotes from Table 1.

* Thyroids were removed and serum collected for ELISAs on the indicated days after cell transfer.

† Sera were obtained on the day of EAT assessment.

Table 5. Treatment of Recipients with Anti-CD4 mAb Suppresses Granulomatous EAT Induced by Cells Cultured with Anti-IL-2R mAb

Exp.	Cells cultured with:	Treatment of recipients*	Severity of EAT [†]						Granulomatous lesions	Anti-MTg IgG [§]	
			0	1+	2+	3+	4+	5+		1/100	1/400
1	MTg	None	0	0	1	1	4	0	2/6	0.608 ± 0.041	0.440 ± 0.044
	MTg	GK1.5 day 4	4	1	0	0	0	0	0/5	0.385 ± 0.066	0.232 ± 0.057
	MTg+M7/20	None	0	0	0	0	0	5	5/5	0.710 ± 0.019	0.697 ± 0.019
	MTg+M7/20	GK1.5 day 4	0	2	3	1	0	0	3/6	0.301 ± 0.040	0.189 ± 0.038
2	MTg*	None	0	5	0	0	0	0	0/5	0.118 ± 0.056	0
	MTg+M7/20*	None	0	1	3	2	0	0	5/6	0.296 ± 0.033	0.035 ± 0.018
	MTg	None	1	0	0	4	0	0	1/5	0.211 ± 0.057	0.103 ± 0.022
	MTg	GK1.5 day 11	3	2	0	0	0	0	0/5	0.163 ± 0.043	0.062 ± 0.013
	MTg+M7/20	None	0	0	0	1	5	0	6/6	0.547 ± 0.058	0.219 ± 0.061
	MTg+M7/20	GK1.5 day 11	2	2	2	0	0	0	2/7	0.379 ± 0.072	0.172 ± 0.025

See footnotes from Table 1.

* Recipients were given 0.3 ml GK1.5 (anti-CD4) ascites 4 d (exp. 1) or 11 d (exp. 2) after cell transfer (4).

† EAT was assessed on day 19 after cell transfer except in the two groups designated with the asterisks, where EAT was assessed 11 d after cell transfer.

§ Sera were obtained on the day of EAT assessment, i.e., day 11 for the two groups indicated by asterisks and day 19 for the other groups.

sions and the single 3+ lesion in the two experiments also exhibited some granulomatous features. In Table 5, exp. 1, the thyroids of untreated recipients of cells cultured with M7/20 (line 3) were essentially totally destroyed and exhibited all of the granulomatous histopathologic features discussed above (Fig. 1, C-G), whereas three of six anti-CD4-treated recipients of these same cells (line 4) showed no evidence of granulomatous changes. Recipients of cells cultured with MTg and M7/20 assessed for EAT 11 d after cell transfer (Table 5, exp. 2) already have granulomatous EAT lesions and increased anti-MTg responses relative to controls, but both autoantibody responses and EAT severity further increase in both groups by day 19. A comparison of EAT severity of mice in line 1 of Exp. 2 (Table 5) with its respective GK1.5-treated counterpart (line 4) indicates, in agreement with our previous observations (4), that GK1.5 treatment can reverse EAT in mice. Comparisons of line 2 vs. line 6 of exp. 2 indicates that this also appears to be true for granulomatous EAT mediated by cells cultured with M7/20. Injection of anti-CD4 mAb early after cell transfer (exp. 1) also resulted in some decrease in the anti-MTg responses, which was most evident for the recipients of cells cultured with MTg and M7/20. However, anti-CD4 mAb had little effect on anti-MTg responses when injected 11 d after cell transfer (exp. 2). These results indicate that CD4⁺ T cells are required to induce both the severe granulomatous EAT lesions and the increased anti-MTg responses in recipients of cells cultured with MTg and M7/20. Moreover, addition of anti-CD4 mAb to cultures with MTg and M7/20 completely prevents activation of cells capable of transferring EAT (data not shown).

IgG Autoantibody Responses. From the results presented above, it is evident that recipients of cells cultured with MTg and M7/20 always produced more anti-MTg autoantibody than controls. As shown in Table 4, these increased anti-MTg

responses were observed in recipients of cells cultured with MTg and M7/20 at all times tested, with maximal anti-MTg IgG responses occurring on days 19–21. Although we have never obtained severe granulomatous EAT lesions in the absence of augmented anti-MTg responses, it is not yet known whether this increased B cell activity contributes directly to the qualitative and/or quantitative differences in thyroid pathology in these mice (see Discussion).

To determine if the increased autoantibody produced by recipients of cells cultured with MTg and M7/20 was due to preferential production of antibody of a particular IgG subclass, ELISAs were developed using antibodies specific for IgG subclasses (Table 6). The anti-MTg responses included antibody of the IgG1, IgG2A, and IgG2B subclasses and all subclasses were increased relative to controls in recipients of cells cultured with M7/20, 3C7, or 7D4. Little or no IgG3 antibody was detected in any of these sera. Thus, the increased IgG autoantibody responses in mice receiving cells cultured with anti-IL-2R mAbs are apparently not due to a preferential increase in antibody of a particular IgG subclass. In other experiments, twofold serial dilutions of sera from recipients of control vs. M7/20-cultured cells were tested in ELISAs in order to more directly compare the amount of anti-MTg produced by the two groups. The dilution at which the OD₄₁₀ reached 0.100 was used as the endpoint. Sera from recipients of cells cultured with MTg alone generally reached this endpoint between a 1:400 and 1:800 dilution of serum, while sera from recipients of cells cultured with MTg and anti-IL-2R mAbs reached this endpoint between 1:3200 and 1:12800.

Flow Cytometric Analysis of Cell-cultured Populations. To determine if cells cultured in the presence of M7/20 had any major alterations in the proportions of various cell populations recovered after 72 h of culture, cells cultured with MTg

Table 6. Subclass Distribution of MTg-specific IgG Autoantibodies

Exp.	Cells cultured with:	MTg-specific IgG*		
		IgG1	IgG2A	IgG2B
1	MTg	0.209 ± 0.051	0.094 ± 0.051	0.003 ± 0.003
	MTg + M7/20	0.468 ± 0.052	0.560 ± 0.072	0.155 ± 0.045
	MTg + 3C7	0.581 ± 0.053	0.636 ± 0.084	0.285 ± 0.094
2	MTg	0.239 ± 0.034	0.319 ± 0.047	0.032 ± 0.016
	MTg + M7/20	0.342 ± 0.028	0.831 ± 0.011	0.392 ± 0.028
	MTg + 7D4	0.389 ± 0.038	0.773 ± 0.036	0.469 ± 0.046
3	MTg	0.349 ± 0.031	0.416 ± 0.023	0.091 ± 0.031
	MTg + M7/20	0.558 ± 0.025	0.664 ± 0.025	0.491 ± 0.039

* Sera obtained 19 d after cells transfer were tested for MTg-specific IgG autoantibodies by ELISA as described in Materials and Methods. In Exps. 1 and 2, sera were tested at a 1:400 dilution, and in Exp. 3, sera from control (MTg) recipients were at 1:100 and those from MTg + M7/20 recipients were at 1:400. Data are mean ± SEM of OD₄₁₀ of five to six serum samples per group.

alone or MTg with M7/20 were examined by flow cytometry. There was no apparent difference in the percentages of Ig⁺ B cells, Thy-1⁺, CD8⁺, or CD4⁺ T cells, or Ia⁺ or IL-2R⁺ cells recovered from the two groups (data not shown). The percentage of IL-2R⁺ cells was quite low for both control and M7/20-cultured populations (10–15% of total cells), and the percentage of IL-2R⁺ cells was even lower at 0, 24, or 48 h of culture. Thus, the effects of M7/20 on EAT and anti-MTg responses cannot be attributed to a major alteration in the proportions of T or B cells or to a significant alteration in the proportion of cells expressing the IL-2R.

Discussion

At least two subsets of murine CD4⁺ T cell clones can be distinguished both functionally and by the particular cytokines they produce after activation by antigen and APC (30–32). One subset, Th1, produces IL-2, IFN- γ , and lymphotoxin, while the Th2 subset produces IL-4, IL-5, and IL-6 (30–32). Th2 are very effective helper cells for B cells, especially for the production of IgE and IgG1 antibody (31–34). While Th1 cells can provide help to B cells, particularly for IgG2A responses (31–34), they are generally less effective helpers than are Th2 cells (31–33, 35, 36). There is some evidence that CD4⁺ T cells corresponding to Th1 and Th2 subsets also exist among noncloned CD4⁺ T cells in vivo and that differential activation of CD4⁺ T cell subsets after immunization may lead to qualitatively distinct immune responses (31, 32, 37–40). In addition, Th1-like cells are reported to be the major proliferating T cells in antigen-primed lymph nodes (32).

The present study demonstrates that when immune spleen cells containing effector T cells for EAT were activated in vitro with MTg in the presence of anti-IL-2R mAb, these cells induced severe granulomatous EAT in recipient mice.

Although MTg was absolutely required to activate cells to transfer granulomatous EAT in conjunction with anti-IL-2R mAb (Table 1, Exp. 2), cell division in vitro apparently was not required since both MTg-specific and mitogen-induced proliferative responses were undetectable in the presence of the M7/20 and 7D4 mAbs (Tables 1 and 2). Since CD4⁺ T cells are required for development of both classical lymphocytic EAT and severe granulomatous EAT (Table 5), activation of different CD4⁺ T cell subsets in the presence vs. the absence of anti-IL-2R mAb could explain our results. It is tempting to speculate that CD4⁺ Th2-like cells induce severe granulomatous EAT accompanied by high anti-MTg autoantibody responses, while Th1-like cells may either induce less severe lymphocytic EAT or suppress activation of Th2-like EAT effector cells. Since both Th1 and Th2 cells express IL-2R and respond to IL-2 (30, 32, 41, 42), anti-IL-2R mAb must preferentially bind to Th1 or inhibit the activation or function of Th1 but not of Th2 cells. Since IL-4 is the autocrine growth factor for Th2 cells (41, 42), blocking of IL-2-induced growth by anti-IL-2R mAb might enable Th2-like cells to be activated by locally produced IL-4. Moreover, IL-4-producing T cells can be selectively expanded by culturing antigen-primed T cells with IL-4 and IL-1 in the presence of anti-IL-2R mAb (43). There is also evidence that in vivo injection of anti-IL-2R mAb suppresses the function of Th1-like cells while having little or no effect on Th2-like cells that provide help to B cells (44). It should also be noted that IL-4-producing Th2 clones can induce EAT in mice (45). One of these clones, B7, induced severe EAT characterized by the striking presence of neutrophils; this clone was very effective in providing help to B cells (22). Moreover, we have found that MTg-sensitized spleen cells cultured with MTg in the presence of mAbs specific for IL-2 and IFN- γ also transfer severe granulomatous EAT and increased anti-MTg autoantibody responses (Mullen, H. B., G. Sharp, J. Bickel, and M.

Kyriakos, manuscript in preparation). Thus, all these observations are consistent with the hypothesis that Th2-like CD4⁺ T cells induce severe granulomatous EAT and Th1-like cells or their products may downregulate the function of these cells. We are currently attempting to derive MTg-specific Th1 and Th2 clones in order to directly test this hypothesis.

The fact that severe granulomatous EAT lesions were always accompanied by increased anti-MTg autoantibody responses raises the possibility that MTg-specific B cells and/or anti-MTg autoantibody may contribute to the pathophysiology of these lesions. While CD4⁺ T cells alone can induce severe EAT resembling in some respects the lesions produced by cells cultured with anti-IL-2R mAb (22; S.J. Stull, unpublished observations), further studies (currently in progress) are required to directly address this issue. It is of interest that the increased anti-MTg responses in recipients of cells cultured with anti-IL-2R mAb are comprised of at least three IgG subclasses, IgG1, IgG2A, and IgG2B, with no apparent preferential increase of a particular IgG subclass (Table 6). This is perhaps unexpected if anti-IL-2R mAb inhibits activation of Th1 cells, since IFN- γ produced by Th1-like cells is presumably required for IgG2A production (31–34). However, since B cells were initially primed in donor mice where activation of Th1-like cells was not prevented, commitment to IgG2A production may have occurred before exposure of the cells to anti-IL-2R mAb.

Finally, the granulomatous EAT lesions that were produced in these studies appear to be similar to those reported for certain strains of mice actively immunized with MTg and adjuvant (46, 47). DeQuervains thyroiditis in man is also characterized by granuloma formation and giant cells, although this disorder is not considered to be autoimmune and may have a viral etiology (47, 48). The fact that granulomatous autoimmune lesions can be reproducibly produced by exposure of sensitized T cells to antigen in the presence of anti-IL-2R mAb provides an excellent opportunity to explore the mechanisms involved in the induction of granulomatous lesions and to compare them with the mechanisms involved in the production of the traditional mononuclear cell EAT lesions. Granulomatous inflammatory lesions are a major pathologic feature of several human diseases, e.g., Wegener's granulomatosis, allergic granulomatosis, giant cell arteritis, and sarcoidosis. The mechanism by which such lesions occur are not well understood, and current treatments with steroids and cytotoxic drugs are nonspecific and not always effective. An animal model of antigen-specific autoimmune granulomatous disease, as described here, should provide an opportunity to explore the mechanism involved in such diseases and to develop more specific therapeutic interventions that might be useful for their treatment.

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