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Constitutive synthesis of tumor necrosis factor in the thymus

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ABSTRACT **Although tumor necrosis factor (TNF) is a major mediator of endotoxic shock, the normal function of TNF that has preserved this protein throughout mammalian evolution remains unknown. If the protein serves a role in normal development or homeostasis, it must be produced under physiologic conditions. To determine whether TNF secretion occurs in normal animals, and to define the tissue sources of the protein, we prepared a reporter construct in which the TNF coding sequence and introns are replaced by the chloramphenicol acetyltransferase (CAT) coding sequence. This construct was inserted into the murine genome, yielding 13 transgenic founders. Macrophages harvested from 4 of the transgenic lines expressed CAT activity after stimulation with *Escherichia coli* lipopolysaccharide *in vitro*. Each of these 4 transgenic lines also constitutively expressed CAT activity in the thymus but in no other tissue examined. Cultured thymocytes secrete TNF, as demonstrated both by cytotoxicity assays and by immunoprecipitation of radiolabeled thymic culture medium. CAT activity was associated with the thymic lymphocyte population and not with thymic macrophages or dendritic cells. CAT activity was present in thymic lymphocytes irrespective of CD4 or CD8 expression; T cells from the spleen, however, had no detectable CAT activity. The biosynthesis of TNF in the thymus of normal animals implies a role for this protein in the development or regulation of the immune response.**

Tumor necrosis factor (TNF) is recognized as a major mediator of endotoxic shock (1, 2), wasting (3–5), and diverse forms of inflammation (6–8). However, it is unknown whether TNF serves a function in the absence of disease (9, 10); indeed, no evidence of TNF production in normal animals has ever been set forth, in part because of the many complexities that arise in detecting its presence. For example, TNF biosynthesis is largely controlled at a translational level (11), so that TNF mRNA is present in a number of tissues without actual biosynthesis of the protein (12, 13). Once TNF mRNA is translated, the protein is efficiently secreted and rapidly degraded (14); thus immunocytochemical detection of TNF is often impossible.

We considered that many of the problems inherent in the detection of TNF might be overcome through the design of a reporter gene that would mimic the behavior of the authentic TNF gene, but which would give rise to a nonsecreted and readily detectable product. Critical to the design of this reporter were data demonstrating the important regulatory role of the TNF 3′ untranslated region (UTR). Previous studies in our laboratory have shown that the TNF 3′ UTR exerts an essential function in macrophages, suppressing translation of TNF mRNA by resting cells but permitting TNF biosynthesis after lipopolysaccharide (LPS) activation (11). In addition, the TNF 3′ UTR was shown to restrict expression of the reporter to certain cell types, whereas the TNF promoter was ubiquitously active (15).

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We recently described a reporter gene construct (CAT_{TNF}), in which a chloramphenicol acetyltransferase (CAT) coding sequence was flanked by the mouse TNF promoter/enhancer region, 5′ UTR, and 3′ UTR. This reporter closely mimicked the behavior of the TNF gene when permanently transfected into mouse macrophages of the RAW 264.7 cell line (16). In this report, we describe the production of transgenic mice that bear the CAT_{TNF} construct and the constitutive expression of this reporter by thymic lymphocytes.

MATERIALS AND METHODS

Generation of Transgenic Mice. The CAT_{TNF} reporter that served as a transgene in these studies is a 3963-base-pair (bp) fragment of DNA that contains the following (5′→3′): the distal coding sequence of lymphotoxin, the enhancer- and promoter-bearing sequence lying between the lymphotoxin and TNF genes, the TNF 5′ UTR (modified by the insertion of an 82-bp polylinker sequence), the CAT coding sequence, and the TNF 3′ UTR and polyadenylylation signal sequence (Fig. 1). Transgenic mice bearing the reporter construct were produced by microinjection of the purified sequence into pronuclei of B6 × SJL zygotes (DNX Corp., Athens, OH). Founders were crossed to BALB/c mice (Sasco, Omaha, NE), and F_1 animals bearing the transgene were identified by dot blotting DNA from tail cuttings.

CAT Biosynthesis in Peritoneal Macrophages. Peritoneal macrophages from 6-week-old mice heterozygous for the CAT reporter were elicited as previously described (17). Macrophages were suspended in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS; GIBCO), then plated at a density of 2×10^6 cells per well in six-well plates. Cells were allowed to adhere for 2 hr, then stimulated with *Escherichia coli* LPS (strain O127:B8; Difco) at a final concentration of 1 μg/ml of medium for the stated period of time. After stimulation, cell lysates were prepared by freezing in 0.25 M Tris·HCl, pH 7.5.

CAT Biosynthesis in Organs. Organs from 6-week-old mice heterozygous for the CAT reporter were removed and homogenized in 1 ml of 0.3 M NaCl/0.03 M sodium citrate/0.4 M Tris, pH 7.4, placed on dry ice for 10 min, then allowed to thaw at room temperature. After centrifugation at 16,000 × *g* for 10 min at 4°C, supernatant protein was quantitated, and samples were diluted in the homogenization buffer to yield a final protein concentration of 1 mg/ml; ovary and thymus samples were diluted to yield a final protein concentration of 200 μg/ml. Samples were then heated to 65°C for 5 min and centrifuged at 16,000 × *g* for 15 min at 4°C, and the supernatants were collected. CAT assays were performed with 100 μl of the supernatant.

Abbreviations: TNF, tumor necrosis factor; LPS, lipopolysaccharide; CAT, chloramphenicol acetyltransferase; UTR, untranslated region.

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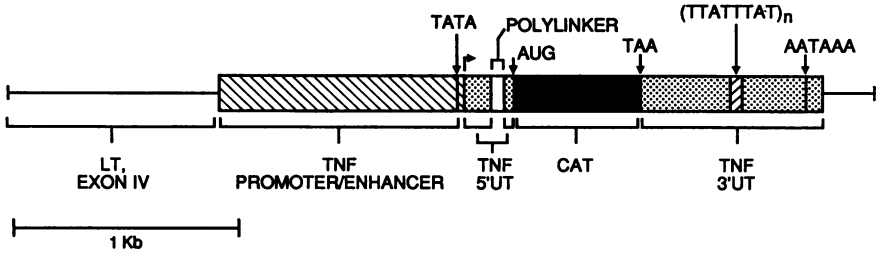

FIG. 1. The CAT construct used as a reporter of TNF biosynthesis. Kb, kilobases; LT, lymphotoxin; hatched bar, TNF promoter/enhancer region; stippled bar, TNF 5′ and 3′ UTRs; and solid bar, CAT coding sequence; the TTATTTAT sequence is represented by the reversed hatched bar in the TNF 3′ UTR.

TNF Synthesis by Cultured Thymocytes. Single cell suspensions of thymocytes from 1-week-old BALB/c mice were incubated in DMEM for 6 hr at 37°C. Medium was then assayed for TNF activity by the L929 cytotoxicity assay as previously described (18). To assure specificity, cytotoxicity was neutralized by preincubation of medium with a TNF receptor-IgG chimeric protein inhibitor (19).

Immunoprecipitation of TNF. Two thymuses from 1-week-old BALB/c mice were minced in DMEM and incubated at 37°C for 6 hr. Medium was harvested, and SDS was added to a final concentration of 1%. The sample was then heated to 100°C for 5 min, then dialyzed against three changes of Dulbecco's phosphate-buffered saline (PBS), pH 7.4. Tris·HCl, pH 7.4, was added to a final concentration of 50 mM. The sample was transferred to a glass test tube coated with 50 μg of Iodo-Gen (Pierce); 1 mCi (37 MBq) of $Na^{125}I$ (Amersham) was added and gently swirled for 15 min. The reaction mix was pipetted into 3 vol of PBS containing bovine serum albumin (Sigma) at 10 mg/ml, 10 mM NaI, and 1 mM 2-mercaptoethanol and was dialyzed first against PBS supplemented with 0.2 M NaCl, 10 mM NaI, and 1 mM 2-mercaptoethanol and then against two changes of PBS containing 1 mM 2-mercaptoethanol. For preabsorption, 100 μl of a 50% suspension of staphylococcal protein A-Sepharose (Sigma) was added, mixed for 1 hr at 4°C, and then removed by centrifugation. The sample was divided into three aliquots. Immunoprecipitation with 40 μl of preimmune serum, rabbit anti-TNF antiserum, or rabbit anti-TNF antiserum plus 20 μg of noniodinated recombinant mouse TNF was carried out for 90 min at 4°C, in a final volume of 0.5 ml, in the presence of 0.25% SDS and 0.5% Nonidet P-40. Then 80 μl of the protein A-Sepharose suspension was added to each sample and mixed for 2 hr at 4°C. After centrifugation, the supernatant was discarded. The complexed protein A-Sepharose was washed at room temperature as follows: three times with 10 mM Tris·HCl, pH 7.4/500 mM NaCl/0.5% Nonidet P-40/0.25% SDS; twice with 10 mM Tris·HCl, pH 7.4/150 mM NaCl/0.5% Nonidet P-40/0.25% SDS; twice with 10 mM Tris·HCl, pH 7.4/0.1 mM EDTA (TE); twice with TE plus 400 mM urea; and twice with TE. The complexed protein A-Sepharose was then boiled for 5 min in 50 μl of 125 mM Tris·HCl, pH 6.8/4% SDS/20% (vol/vol) glycerol/1.4 M 2-mercaptoethanol/0.002% bromphenol blue. Samples were resolved on a 14–18% gradient SDS/polyacrylamide gel.

Thymocyte Separations. *Density gradient.* Thymocytes from 2-week-old mice heterozygous for the CAT reporter were suspended in 35% bovine serum albumin and allowed to migrate in a discontinuous albumin gradient as previously described (20).

Expression of CD4/CD8. Suspensions of nonadherent thymocytes were labeled with rat monoclonal antibodies against mouse CD4 or CD8 (Biotec Labs, Houston). Cells were then selected by adherence to plastic beads coated with goat anti-rat IgG (Biotec Labs). Selected and nonselected cells were counted and assayed for CAT activity.

Thymic and Splenic T Cells. Thymuses and spleens were removed from 2-week-old mice heterozygous for the CAT reporter. Suspensions of cells from pooled spleens and thymuses were passed over a Ficoll gradient to isolate monocytes and lymphocytes. To remove B cells and macrophages, the cell suspensions were then passed over a column consisting of plastic beads coated with goat anti-mouse IgG (Biotec Labs). Samples containing 6×10^7 thymic and splenic T cells were assayed for CAT activity.

CAT Assays. CAT activity was determined by the thin-layer chromatography procedure of Gorman *et al.* (21). For thymocyte selection experiments, CAT activity was quantitated by counting acetylated forms of [^{14}C]chloramphenicol by phosphor-imager (Molecular Dynamics, Sunnyvale, CA), and related to milliunits of CAT activity from a purified bacterial CAT standard (Pharmacia).

RESULTS AND DISCUSSION

Table 1 presents the expression characteristics determined for each transgene. Seven of the transgenes had been silenced in all tissues examined, including macrophages, whether or not LPS had been added to the culture. In four of the transgenic lines, CAT was inducibly expressed within peritoneal macrophages after challenge with LPS, which accurately reflected TNF biosynthesis by these cells. In all four of these transgenic lines, CAT was constitutively expressed in the thymus of normal 6-week-old animals and in none of the other organs sampled (Fig. 2). CAT activity was also found in the thymus of newborn, 1-week-old, and 3-week-old transgenic mice, but in no other organ tested. Of the nine lines in which CAT biosynthesis was not inducible by LPS, one line expressed CAT in the lung, and one line expressed CAT in all tissues examined.

Table 1. CAT activity in transgenic lineages containing the CAT_{TNF} construct

Transgene lineage no.	LPS induction of CAT synthesis in macrophages	Constitutive expression
1	+	Thymus
2	+	Thymus
3	+	Thymus
4	+	Thymus
5	−	−
6	−	−
7	−	−
8	−	Lung
9	−	−
10	−	−
11	−	−
12	−	−
13	−	All tested

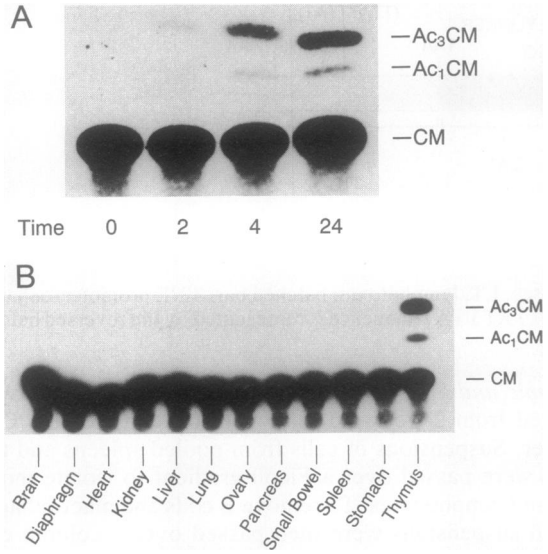

FIG. 2. (*A*) Induction of CAT biosynthesis by LPS. Peritoneal macrophages from transgenic mice were exposed to LPS *in vitro* for 2, 4, or 24 hr. CAT activity from macrophages not exposed to LPS is indicated by 0. CM, nonacetylated chloramphenicol; Ac_1CM and Ac_3CM, the mono- and triacetylated forms of chloramphenicol produced by CAT. (*B*) Constitutive CAT biosynthesis in the thymus. Organs were removed and processed for CAT assay. All samples contained 100 μg of protein prior to heat inactivation, except for the ovary and thymus samples, which contained 20 μg of protein. The CAT assay is depicted as in the previous figure.

Since four independent transgenic lines expressed CAT within the thymus, we reasoned that TNF might also be constitutively synthesized within this tissue. In an attempt to detect secretion of the TNF molecule itself, single cell suspensions of thymocytes from 2-week-old mice were incubated for 6 hr *in vitro*. TNF was detected in the culture medium (65–90 pg per thymus) by the L929 cytotoxicity assay. The cytotoxicity was abolished by preincubation of culture medium with a TNF receptor-IgG chimeric protein inhibitor. Furthermore, when thymocyte culture medium was radiolabeled with ^{125}I and immunoprecipitated by using rabbit anti-mouse TNF antibody, a secreted 17-kDa TNF species, as well as higher molecular weight proteins previously identified as glycosylated forms of TNF (18, 22), could be identified on an SDS/polyacrylamide gel. No such proteins were apparent when nonimmune serum was used to immunoprecipitate thymic secretory products or when an excess of unlabeled recombinant mouse TNF was added to the immunoprecipitation system (Fig. 3).

To determine whether CAT biosynthesis occurred within lymphocytic or macrophage/dendritic cells of the thymus, single cell suspensions of thymic origin were fractionated on a discontinuous albumin gradient and allowed to adhere to plastic surfaces as previously described (20). Only nonadherent cells from lymphocyte-enriched bands (bands 1 and 2) expressed the CAT reporter. No CAT activity was evident in the adherent population (band 3), consisting chiefly of macrophages and dendritic cells (Fig. 4*A*). Additional selection of thymic lymphocytes indicated that CAT activity was present in these cells whether or not they expressed CD4 or CD8 molecules (Fig. 4*B*). Although it is possible that the CAT reporter is activated during T-cell selection, T cells isolated from the spleen fail to express CAT (Fig. 4*C*). Additional studies designed to detect CAT in fixed, cell-sorted lymphocytes will be necessary to eliminate all possibility of CAT activation during separation.

Several cell types synthesize TNF upon activation by various stimuli (23–26). Kinkhabwala and coworkers (27)

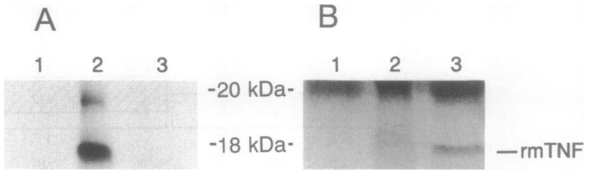

FIG. 3. Immunoprecipitation of TNF. (*A*) Autoradiogram of immunoprecipitation gel. (*B*) Coomassie-stained gel. Lane 1, control antiserum; lane 2, TNF antiserum; lane 3, TNF antiserum plus nonradiolabeled recombinant mouse TNF. (*A*) A 17-kDa protein species, which corresponds in mobility to recombinant murine TNF, and higher molecular weight TNF "ladder" proteins are precipitated by anti-TNF antiserum (lane 2). These bands are not observed when nonradiolabeled recombinant murine TNF is added to the immunoprecipitation mix (lane 3). (*B*) Coomassie stain of the same gel demonstrates immunoprecipitation of the nonradiolabeled recombinant murine TNF (rmTNF). The Coomassie-stained bands at the top of the gel represent immunoglobulin light chains from the antiserum used for immunoprecipitation.

have demonstrated that T cells express a membrane-associated form of TNF after activation by phorbol esters and ionomycin. Recently Salgame *et al.* (28) have shown that T-cell clones from humans with leprosy secrete TNF and other cytokines upon stimulation *in vitro*. Although TNF production has not been demonstrated *in vivo* in normal animals, many workers have nonetheless speculated that TNF may act to influence development of the normal immune system (29). If this view were correct, one must necessarily detect TNF biosynthesis *in vivo* during at least a part of the ontogeny of the immune system. The CAT_{TNF} reporter described here has revealed that TNF is indeed synthesized in the thymus of normal animals. The consistent pattern of thymic transgene expression, as well as the detection of TNF secreted by cultured thymocytes, suggests a physiologic function of TNF, distinct from its role in sepsis and other pathologic states. TNF expression occurs only in an evolving T-cell population, since mature T cells present in spleen do not synthesize CAT. At least a fraction of thymic TNF is secreted; however, we are uncertain whether the protein exerts its effects locally or is active at a distance.

Previous studies have indicated that TNF may influence T cell function in several ways. Palladino and coworkers (29) demonstrated that murine thymocyte proliferation is stimulated by mouse (but not human) TNF *in vitro*, and they therefore speculated that TNF may be involved in thymocyte proliferation *in vivo*. In addition, Shalaby *et al.* (30) showed that TNF stimulates the mixed lymphocyte reaction (MLR), whereas anti-TNF antibodies strongly inhibit the MLR *in vitro*. It is therefore possible that the function of TNF within the thymus is to influence thymocyte proliferation and/or selection, either directly or by enhancing the signals from other lymphokines. The proliferative response to TNF has recently been shown to be transmitted by the 75-kDa TNF receptor, which is present on both T and B lymphocytes.

de Kossodo and coworkers† have recently shown that passive immunization of neonatal mice with antibodies against TNF results in thymic involution, lymphoid hypoplasia, and runting. Considering this observation along with data presented here, it is possible that antibodies against TNF interfere with a normal TNF function within the thymus, leading to profound disturbances in lymphocyte maturation and overall development of the organism.

Alternatively, it is possible that TNF may participate in the development of self-tolerance by contributing to the deletion

†Grau, G., de Kossodo, S., Pointaire, P., Ody, C., Bonnefoy, J. Y., Piguet, P. F. & Vassalli, P., Third International Conference on TNF and Related Cytokines, Nov. 21–25, 1990, Makuhari, Chiba, Japan, Poster Presentation 4A–22, (p. 190).

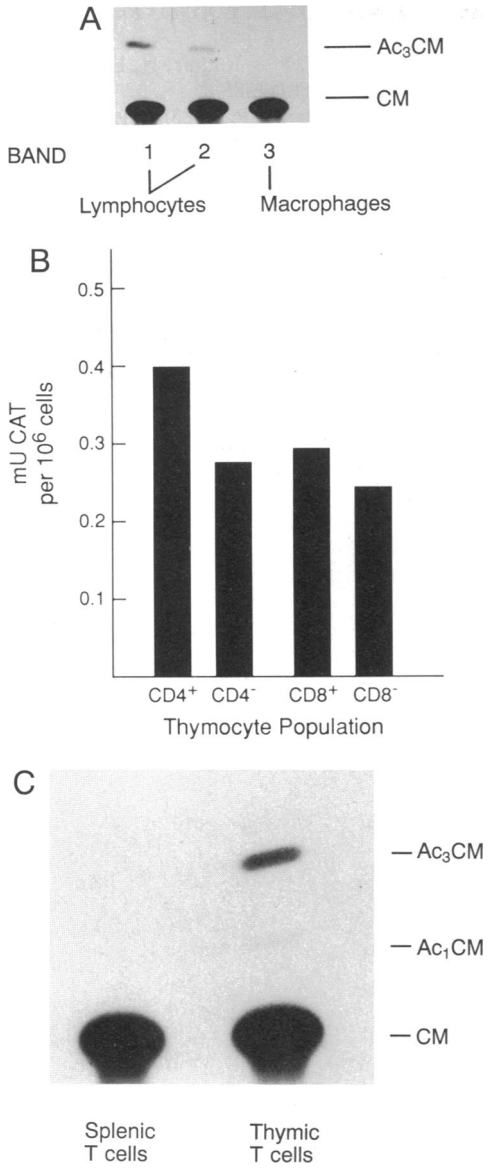

FIG. 4. (*A*) CAT biosynthesis by thymic lymphocytes. Band 1 (interface between 35% and 29% bovine serum albumin) and band 2 (interface between 29% and 27% albumin) represent relatively immature and mature lymphocyte populations, respectively. Macrophages and dendritic cells from band 3 (interface between 27% and 10% albumin) were further enriched by allowing adherence to plastic. CAT assays are depicted as in previous figures. (*B*) CAT biosynthesis by thymic lymphocyte subpopulations. $CD4^+$, $CD4^-$, $CD8^+$, and $CD8^-$ thymic lymphocytes were selected as described in the text. The CAT activity manifested by each population was quantitated by counting the acetylated chloramphenicol bands by computerized phosphor-imaging, and related to a CAT standard to determine milliunits (mU) of CAT activity. CAT activity in each population is normalized per 10^6 cells in that fraction. (*C*) CAT biosynthesis by thymic and splenic T cells. Thymic and splenic T cells (6×10^7 each) from transgenic animals were tested for CAT activity. The CAT assay is depicted as in the previous figures.

of autoreactive clones within the thymus (31–33). Indeed, TNF is known to cause the death of its cellular targets by apoptosis (34), a mechanism implicated in lymphocyte deletion during thymic education (35). The apoptotic response to TNF, witnessed in certain transformed cell lines, appears to be transmitted exclusively through the 55-kDa TNF receptor (36). The runting and lymphoid hypoplasia witnessed in passively immunized animals might thus reflect the development of an autoimmune process secondary to the survival of autoreactive clones. Similar runting syndromes have been described as a consequence of neonatal graft-versus-host disease (37) or after neonatal thymectomy (38), which itself is characterized by an autoimmune syndrome (39, 40). Jacob and McDevitt (41) have suggested that abnormally low TNF production might lead to autoimmunity, since macrophages from $(NZB \times NZW)F_1$ hybrid mice underproduce TNF in response to endotoxin.

Further work will be required to decipher the function of TNF within the developing thymus and to determine whether human syndromes characterized by abnormalities in growth and lymphoid maturation may arise from disordered TNF regulation. Genetic constructs similar to the one that we have employed, incorporating the regulatory regions of other cytokine genes, might elucidate the roles played by these factors in physiologic and pathologic states.

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