Tumor Necrosis Factor α Is Involved in Mouse Growth and Lymphoid Tissue Development

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Summary

Tumor necrosis factor α (TNF-α), a major mediator of inflammation, also possesses a wide pleiotropism of actions, suggesting its involvement in physiological conditions. TNF-α mRNA is present in mouse embryonic tissues and also in fetal thymus and spleen. Repeated injections of a monospecific polyclonal rabbit anti-mouse TNF-α antibody in mice, starting either during pregnancy or at birth, led to a severe but transient growth retardation, already present at birth, reaching a 35% decrease in body weight at 3 wk, with complete recovery at 8 wk. The insulin growth factor I (IGF-I) blood levels were decreased to about 50%; growth hormone release and other endocrine functions were unaltered. A marked atrophy of the thymus, spleen, and lymph nodes was also observed, with lymphopenia and impaired development of T and B cell peripheral lymphoid structures. The pathways involving TNF-α in IGF-I release and early body growth are probably distinct from those by which TNF-α participates in early development of lymphoid tissues, where its low physiological release may contribute to enhance lymphoid cell expansion.

TNF-α, a cytokine that is produced in the largest amounts by activated macrophages but that can also be released by a wide variety of other cells, is one of the major mediators of inflammation and is involved in a number of infectious and parasitic diseases (1–4). It is also a very pleiotropic agent acting on numerous tissues, and thus is suspected to participate in various physiological functions (2–4). TNF mRNA has been detected in mouse embryos (5), in human fetal thymus (6), and in both extraembryonic and maternal tissues during pregnancy (7). Biologically active TNF has been detected in the amniotic fluid as well as in supernatant culture media from human placental and decidual explants (8, 9). To investigate the possibility that TNF is involved in some developmental steps, this study used the approach of repeatedly injecting pregnant mice or pups at birth with high amounts of a monospecific neutralizing rabbit anti-murine TNF antibody.

Materials and Methods

Antibodies. IgG fractions of either rabbit antiserum to mouse TNF-α or normal rabbit serum were prepared by protein A–Sepharose chromatography (Pharmacia Fine Chemicals, Uppsala, Sweden). These fractions were ultracentrifuged at 150,000 *g* for 150 min, and only the upper third, deaggregated fractions were used to induce and maintain tolerance to rabbit IgG. When affinity-purified anti-TNF antibody was used, it was obtained by elution with 0.58% acetic acid in 150 mM NaCl from a column containing 1 mg recombinant mouse TNF (gift of B. Allet, Glaxo I.M.B., Geneva, Switzerland) coupled to Affigel 10 (Bio-Rad Laboratories, Richmond, CA), after prior washing with 2 M NaCl, followed by dialysis against PBS, pH 7.4. Four different batches of anti-TNF antibodies (obtained from the sera of different rabbits) were used in these experiments.

In Vivo Treatments. Pregnant outbred NMRI mice were obtained from IFFA-Credo (Lyon, France), housed in our facilities, and kept under a laminar flow. Neomycin was added to drinking water. Mice were injected intraperitoneally with either 1 mg of anti-TNF IgG or normal (n)IgG, or with 50 μg of affinity-purified anti-TNF antibody or nIgG. Two injection schedules were used. (*a*) Starting on days 11, 13, and 15 of pregnancy, the offspring were injected every other day with the same amounts of antibody or nIgG. (*b*) Starting on day 2 after birth, neonates of the same litter were injected with antibody or nIgG every 3 d.

Hormonal Assays. Mice were decapitated between 9 and 10 a.m. in conditions ensuring the least possible stress. Insulin growth factor I (IGF-I)[1] and growth hormone (GH) serum levels were measured by specific RIAs using kits provided by NIAMDD, National Pitu-

[1] *Abbreviations used in this paper:* AP, anterior pituitary gland; GH, growth hormone; GHRF, growth hormone–releasing factor; IGF-I, insulin growth factor I.

itary Agency. For IGF-I RIA (10), antibody was generously provided by Dr. L. Underwood (University of North Carolina, Chapel Hill, NC), and standard by Kaby Pharmacia Peptide Hormones (Stockholm, Sweden). For GH RIA, antibody and standard were supplied by the National Institutes of Health (Bethesda, MD), except for the first antiserum, which was kindly given by Dr. Y. Sinha et al. (11). For the study of GH release in vitro, four anterior pituitaries from anti-TNF antibody-treated mice and four from nIgG control mice were packed in 2-ml plastic syringes superfused (0.35 ml/min) with medium only (Earle's balanced salt solution + HSA) or containing various concentrations of GH-releasing factor (GHRF) as described (12). 3-min fractions were collected and GH concentrations were determined by RIA. For the determination of corticosterone blood levels, blood samples were centrifuged at low speed, and the plasmid was stored frozen (−20°C) until direct RIA (13).

Hematological Assays. Differential white blood cell (WBC) counts were performed on Giemsa-stained blood smears. Colony-forming cell assays in the presence of IL-3 and erythropoietin were performed as described (14).

Histology. Histological sections of tissues obtained at autopsy were stained with hematoxylin and eosin. Photographs were taken with a photomicroscope (Carl Zeiss, Inc., Oberkochen, Germany).

Northern Blot Analysis. Detection of TNF-specific mRNA was performed by Northern blot analysis using a radiolabeled pSP65-TNF cRNA probe after glyoxylation of 6 μg total RNA, separation by agarose gel electrophoresis, and transfer to Biodyne membranes (Pall Ultrafine Filtration Corporation, Glen Cove, NY), followed by prehybridization and hybridization conditions as described (15). Filters were exposed to Kodak XAR-5 films at −70°C with intensifier screens (Du Pont Cronex, Boston, MA).

Statistical Analysis. Significance analysis between results obtained from various groups was performed by using the nonparametric Mann-Whitney U-test. Probability values >5% were considered significant.

Results and Discussion

Pregnant mice received rabbit anti-TNF antibody, either as a total IgG fraction or as affinity-purified antibody, or normal rabbit IgG as control, on days 11, 13, and 15 of pregnancy. At birth, newborn mice of anti-TNF antibody-treated mothers were consistently smaller (Fig. 1 *A*), with an average weight of 1.4 ± 0.3 g (mean ± SD; $n = 24$) vs. 1.7 ± 0.5 g ($n = 23$) ($p < 0.001$). In a typical experiment, four of the pups in each group were killed for blood sampling and histological examination, with 16 others receiving intraperitoneal injections of the same amount and type of IgG as given to their mothers, every other day. Eight of these mice were killed at 9 d, and eight at 21 d of age, at which time the anti-TNF antibody-treated mice weighed on average 34% less than their matched controls (Fig. 1, *A* and *B*, at 3 wk of age; $p < 0.01$). A comparable difference in size, as assessed by measurement of the rump-to-tail and tail lengths, was revealed (a 25% decrease average in 3-wk-old anti-TNF antibody-treated mice) ($n = 8$ per group; $p < 0.01$). This protocol was repeated four times with consistent results. To investigate whether the observed effects on growth were permanent, after 3 wk of treatment with anti-TNF antibody, mice were divided into two groups: one ($n = 8$) continued to receive the anti-TNF antibody as described for an additional 3 wk, and in the other group ($n = 8$), the injections were discontinued. From day 21 to 43, no significant difference in body weight (Fig. 1, *A* and *C*) or size (not shown) was observed between the two groups, previously treated with anti-TNF antibodies or still on treatment, although there was still at 43 d a statistical difference ($p = 0.03$) with mice receiving nIgG only. At 58 d, however, controls and mice that had stopped receiving the anti-TNF antibodies 2 wk earlier no longer showed a significant difference in weight (Fig. 1 *A*).

To exclude an effect related to an interference with placental development, a similar schedule of injections was started after birth, on the second day of life. At 7 d, the difference in weight of anti-TNF antibody-treated (3.1 ± 0.4 g; $n = 14$) and control pups (4.4 ± 0.3 g; $n = 12$) was highly significant ($p < 0.001$), and at 3 wk, it was comparable to that shown in Fig. 1 *A*. Combining these two protocols of injections, starting during pregnancy or after birth, a total of 103 anti-TNF antibody-treated mice (44 with IgG fraction and 59 with purified antibody) were compared and killed at various intervals between 2 and 58 d of age. The average weight in 3-wk-old anti-TNF antibody-treated mice (11.4 ± 3.2 g; $n = 42$) was 37.5% less than in controls (18.3 ± 3.2 g; $p < 0.001$). Taken together, these results show that: (*a*) anti-TNF antibody treatment affects somatic growth already in utero, but is also effective when begun immediately after birth; and (*b*) the effect of the antibody treatment is reversible with time. Except for the alteration of the lymphoid organs, described below, gross and histological examination of liver, lung, kidney, adrenals, heart, and pituitary were comparable in both groups, with no detectable evidence of acute or chronic infection in the smaller mice.

To search for possible endocrine and metabolic alterations associated with this growth retardation, the levels of IGF-I were first explored in the pooled sera of three 2-d-old pups from an anti-TNF antibody-treated mother and of three 3-wk-old mice treated since birth. The IGF-I serum levels were, respectively, 43% and 51% those of matched controls. To extend these observations, IGF-I and GH levels were measured in the sera of 21-, 43-, and 58-d-old mice (Table 1). While no difference was observed in GH levels at any age, the IGF-I levels were again markedly decreased in anti-TNF antibody-treated mice at 3 wk. This difference, although less pronounced, was still persistent at 43 and 58 d, when anti-TNF antibody-treated mice had regained a weight and size comparable to that of controls. All groups of mice presented comparable GH contents of the anterior pituitary gland (AP), and comparable GH release in vitro in response to GHRF (not shown), except in 43-d-old mice, where the AP response to 10^{-7} M GHRF was significantly higher in anti-TNF antibody-treated mice (400 ± 9 pg/ml) than in controls (150 ± 22 pg/ml; $p < 0.05$). Since this was the time when treated mice started recovering a normal weight, this raised the possibility of a role of GH in this catch-up phenomenon. Corticosterone levels of treated mice were not significantly different from those of controls at 9 (0.7 ± 0.1 vs. 0.4 ± 0.02 μg; $n = 8$), 21 (10.2 ± 1.8 vs. 6.2 ± 1.4 μg; $n = 8$), or 43

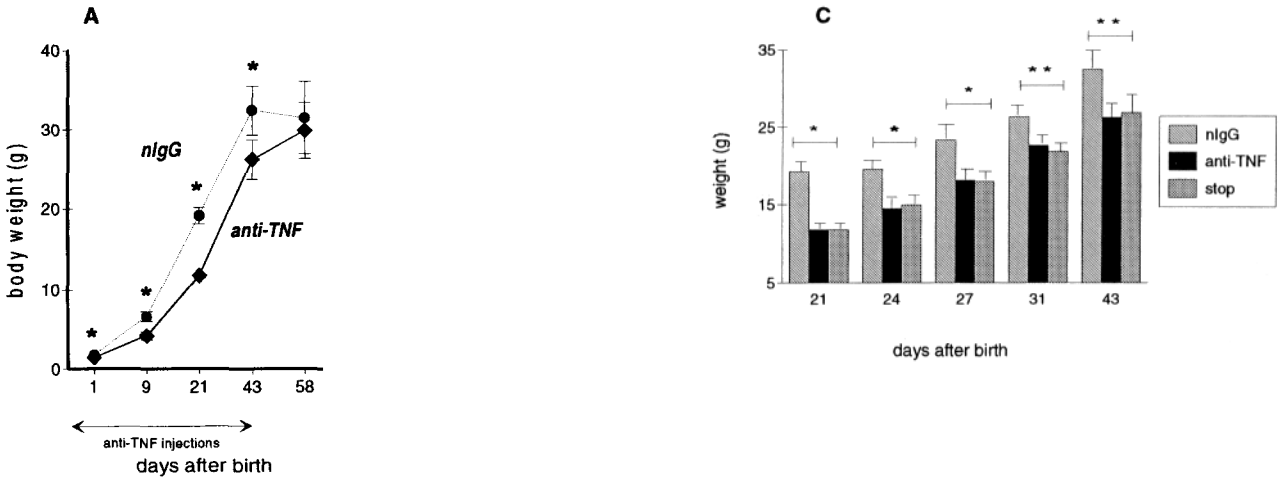


Figure 1. Effect of anti-TNF antibody treatment on growth. (*A*) Growth curve of anti-TNF antibody-treated and control mice between birth and 58 d of age. Significance analysis between results obtained from various groups was performed by using the nonparametric Mann-Whitney U test. Values <5% were considered significant (*). (*B*) A 3-wk-old mouse treated with anti-TNF antibody was smaller than its littermate treated with nIgG (9.1 vs. 17.2 g). (*C*) At 3 wk of age, anti-TNF antibody-treated mice were divided in two groups. One group continued to receive anti-TNF antibody following the same schedule as described for an additional 3 wk (anti-TNF), and the second group received nIgG instead (*stop*). No difference in body weight or size was observed between the two groups. A significant difference was noted between controls and mice previously treated or still on treatment: (*) $p < 0.01$; (**) $p < 0.05$). Persistance of anti-TNF antibody activity in serum was found to range between 72 and 1,200 neutralizing units/ml 4 d after the last injection.

Table 1. *Effect of Anti-TNF Antibody Treatment on GH and IGF-1 Levels*

Day	Treatment	GH	IGF-I
		ng/ml	*ng/ml*
21	nIgG	3.9 ± 0.3	465.55*
	Anti-TNF	4.8 ± 0.5	235.90*
43	nIgG	3.1 ± 0.05	463.47‡
	Anti-TNF	3.4 ± 0.13	322.18‡
58	nIgG	4.1 ± 0.7	452.56‡
	Anti-TNF	4.3 ± 0.5	359.52‡

* Pool of eight mice.
‡ Pool of four mice.

d (11.2 ± 2.6 vs. 6.6 ± 2.6 μg; $n = 4$), as were ACTH contents of the AP and ACTH release in vitro in response to corticotropin-releasing hormone (not shown). This exploration was performed since TNF-α has been shown, under certain conditions, to modulate the corticotroph axis (16). Since, in vitro, TNF has been shown to stimulate hexose transport and to increase the level of glucose-transporter (Glut-1) mRNA expression (17), an effect that might affect growth, the levels of Glut-1 mRNA were analyzed in the striated muscle of 1-wk-old pups of mothers having received anti-TNF antibody ($n = 6$) or nIgG during pregnancy; no difference was found (not shown). Finally, no difference was observed in blood glucose levels between anti-TNF antibody-treated and control mice.

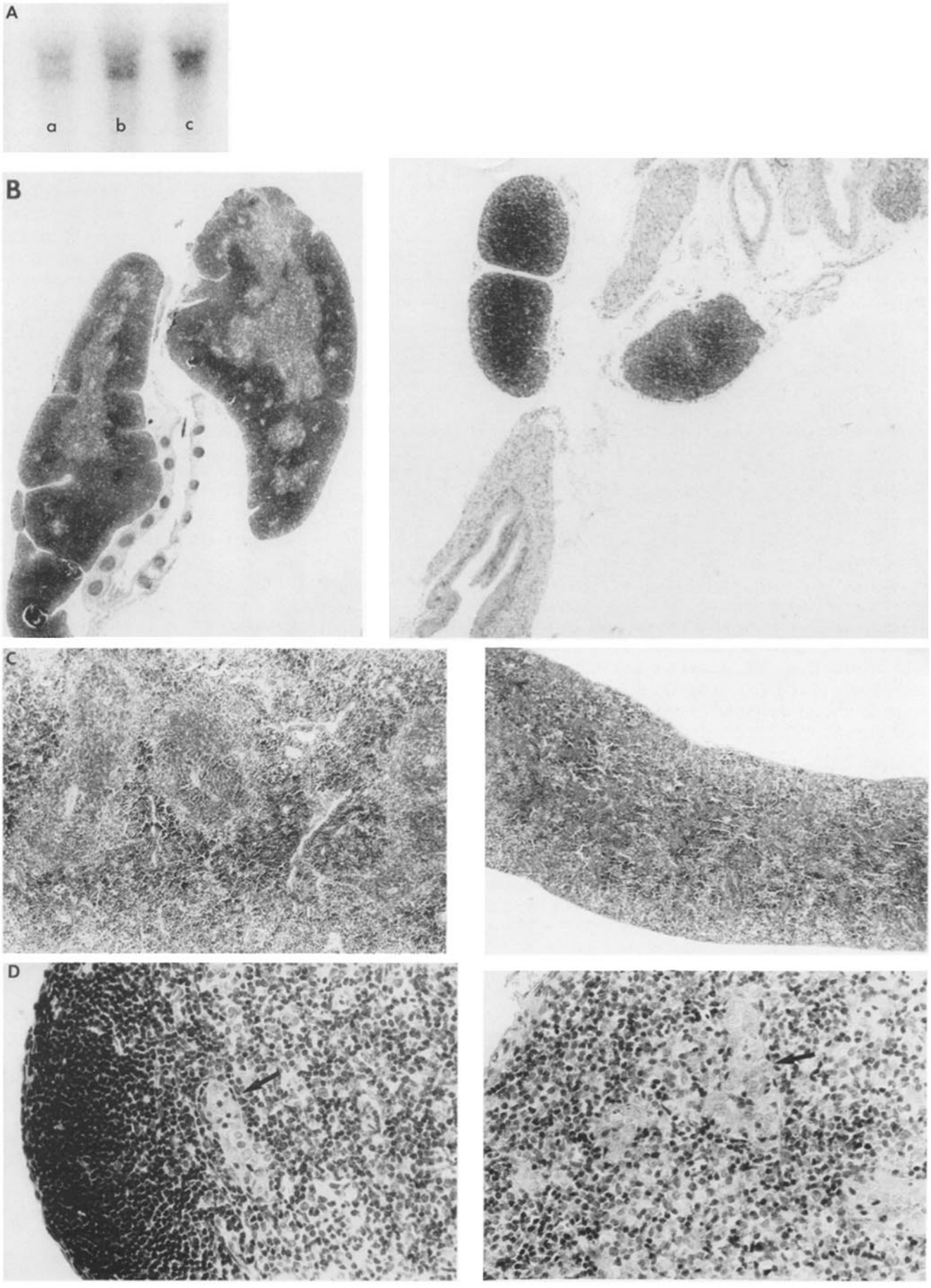
Thus, the only endocrine disturbance detected in anti-TNF antibody-treated mice that appears to be related to their retarded growth is the prolonged decrease in IGF-I serum

levels. Results of the treatment when started at birth rule out the possibility of a deleterious effect on placental growth. The decreased size at birth as well as the absence of gross histologic signs of infection argue against an explanation based on a higher vulnerability to infectious agents, which could result from a lack of TNF and from the impaired development of the lymphoid system described below. Most importantly, infection leads to elevated corticosterone levels, which were not observed in anti-TNF antibody-treated mice. Hyponutrition, known to decrease IGF-I levels (18), would not explain the intriguing discrepancy observed on the eighth week between persisting low IGF-I levels and resumption of normal body weight. Signs of hepatic disease, which can cause decreased IGF-I production (19), were not seen on histologic sections of the liver. Thus, it appears that TNF influences the somatotroph axis through mechanisms not yet understood. The observation that the marked decrease in serum IGF-I levels in anti-TNF antibody-treated mice was associated with normal GH levels (and release in vitro) is consistent with the notion that GH has no effect on fetal and early postnatal growth (20), in which IGF-I and -II appear to play a major role (21).

We have detected TNF mRNA in the thymus and spleen on the 15th day of fetal life and at birth (Fig. 2 *A*), which suggests the possibility of an involvement of TNF in lymphoid organ development. Indeed, the only conspicuous internal alterations shown by the anti-TNF antibody-treated mice at death were those of the lymphoid organs. The thymuses of 2-d-old anti-TNF antibody-treated mice were very small and histologic examination showed a poorly developed medulla, in striking contrast with the thymic structure of control mice (Fig. 2 *B*). On day 9, the average decrease in thymus weight of the anti-TNF antibody-treated mice was 56% (28.7 ± 1.2 mg, $n = 8$ vs. 65.6 ± 5.6 mg, $n = 8$; $p < 0.001$), and on day 21 was 49% (104 ± 4.3 mg, $n = 5$ vs. 204 ± 13.6 mg, $n = 5$; $p = 0.01$). At this age, however, no difference in histologic structure other than size was seen; flow cytometry analysis of thymocyte suspensions showed that the percentages of $CD4^+/CD8^+$, $CD4^-/CD8^-$, and more mature $CD4^-/CD8^+$, $CD4^+/CD8^-$ were comparable in both groups of mice (not shown). The spleens of anti-TNF antibody-treated mice were also smaller, with an average decrease in weight of 39% on day 9 (30.0 ± 1.7 mg, $n = 8$ vs. 48.6 ± 2.8, $n = 8$; $p < 0.001$) and of 33% on day 21 (80 ± 1.5 mg, $n = 5$ vs. 120 ± 6.5 mg, $n = 5$; $p = 0.002$). Histologic examination showed a poorly developed or absent white pulp, in contrast to control mice, a feature apparent on day 9 (Fig. 2 *C*). Flow cytometry analysis of spleen cell suspensions revealed that the percentage of $sIgM^+$ cells for 4-wk-old anti-TNF antibody-treated mice was decreased as compared with controls (16.7 ± 2.6%, $n = 4$ vs. 34.7 ± 8.6%, $n = 4$; $p = 0.01$), a difference accentuated when these values were calculated in total cell number (78% decrease of $sIgM^+$ cells in anti-TNF antibody-treated mice; $p = 0.03$). Histological examination of the axillary and inguinal lymph nodes from 3-wk-old mice revealed in the antibody-treated mice a decreased cellularity and a lack of well-formed cortical primary follicles (Fig. 2 *D*). The specialized postcapillary high endothelial venules from which lymphocytes migrate from blood into lymph nodes were more difficult to find and had, when distinctly seen, an unusual flat endothelial lining (Fig. 2 *D*), probably as a reflection of decreased lymphocytic traffic and cytokine action on the differentiation of these endothelial cells. A marked lymphopenia was observed in the blood of anti-TNF antibody-treated mice, both relative (46.1 ± 6.5 vs. 69.6 ± 5.7% in controls, $n = 5$ per group; $p < 0.05$) and absolute (7.5 ± 2.4 vs. 31.2 ± 5.1 × 10^3 lymphocytes/mm^3 in controls; $p < 0.05$). No conspicuous effect on hematopoiesis was seen since: (*a*) RBC or platelet counts were similar in both groups of mice; (*b*) using colony-forming cell assays in the presence of IL-3 and erythropoietin, similar hematopoietic progenitor numbers were found in the bone marrow or spleen cells of anti-TNF antibody-treated and control mice (not shown).

Since the thymus contains TNF mRNA, and also protein (22), very early in its development, and since TNF acts in vitro as a growth factor for thymocytes (23), in particular in association with IL-2 (24), the effect of anti-TNF antibody is probably exerted locally. Presence of TNF within the thymus may be required for optimal thymocyte proliferation, at least during the period of rapid thymus growth, and for the normal colonization of peripheral lymphoid organs by T lymphocytes. However, the severe alterations of the spleen and peripheral lymph nodes encountered cannot be explained solely on the basis of a depletion in T lymphocytes, as shown by the decreased number of splenic B lymphocytes and the poor development of lymph node primary follicles, which are B cell structures preserved in athymic nude mice. It seems possible that a local production of TNF is required in sites other than the thymus, such as the spleen, to allow a normal process of B cell expansion. TNF has been reported to enhance B cell proliferation in vitro (25, 26). This may also take place in indirect ways, through the stimulation of accessory cells or the production of other cytokines involved in a network necessary for the normal development of the lymphoid system, but not required for that of hematopoiesis. SCID mice have a profound atrophy of lymphoid organs, due to their failure to produce T and B lymphocytes (27), but no obvious impairment of early body growth. The effect of TNF on early body growth is probably exerted through mechanisms distinct from those acting upon lymphoid tissue development, and which remain to be elucidated.

Figure 2. (*A*) Detection of TNF transcripts by Northern Blot analysis in day 15 fetal thymus (lane *a*), spleen (lane *b*), and postnatal day 1 thymus (lane *c*); 6 μg total RNA. (*B*) Marked decrease in size and medullar depletion observed in the thymus of a 2-d-old pup from an anti-TNF antibody-treated mother (*right*, actual length of thymic lobe: 0.8 mm) as compared with control littermate (*left*, length of thymic lobe: 4 mm) (×9). (*C*) Lack of developed splenic white pulp in a 9-d-old anti-TNF antibody-treated mouse (*right*); the spleen is much smaller than that of a control, in which the white pulp is distinctly seen (×6). (*D*) Comparable cortical regions of axillary lymph nodes show general lymphocyte depletion, lack of cortical follicles, and postcapillary venules displaying flat endothelial cells (*arrow*) in 3-wk-old mice treated with anti-TNF since birth (*right*), as compared with control (*left*, normal postcapillary venule with high endothelial cells [*arrow*]). (×23).



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