the loose connective tissue between the macrophage, which reacts to infectious and immunologic stimuli, and the adipocyte, whose energy stores must be mobilized at times of physiologic stress. That these events are reversible with the removal of cachectin suggests approaches that may have potential therapeutic implications for humans. Further characterization of cachectin and its receptor will help to clarify the nature of signal transduction to the adipocyte nucleus.

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Passive Immunization Against Cachectin/Tumor Necrosis Factor Protects Mice from Lethal Effect of Endotoxin

Abstract. *A highly specific polyclonal rabbit antiserum directed against murine cachectin/tumor necrosis factor (TNF) was prepared. When BALB/c mice were passively immunized with the antiserum or with purified immune globulin, they were protected against the lethal effect of the endotoxin lipopolysaccharide produced by* Escherichia coli. *The prophylactic effect was dose-dependent and was most effective when the antiserum was administered prior to the injection of the endotoxin. Antiserum to cachectin/TNF did not mitigate the febrile response of endotoxin-treated animals, and very high doses of endotoxin could overcome the protective effect. The median lethal dose of endotoxin in mice pretreated with 50 microliters of the specific antiserum was approximately 2.5 times greater the median lethal dose for controls given nonimmune serum. The data suggest that cachectin/TNF is one of the principal mediators of the lethal effect of endotoxin.*

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Mammals infected with gram-negative bacteria often develop a state of shock, which is characterized by hypotension, disseminated intravascular coagulation, and renal, hepatic, and cerebral injury. Many of these deleterious consequences of infection can be reproduced in animal models by injecting endotoxin, a lipopolysaccharide (LPS) component of the cell walls of certain bacteria. While the mechanism of action of LPS remains obscure, it is believed that the toxic effects are mediated by factors produced by host cells. Adoptive transfer experiments with LPS-resistant (C3H/HeJ) and -sensitive (C3H/HeN) congenic mice have implicated cells of hematopoietic origin and, in particular, monocytes, as the source of these mediators (*1, 2*).

Recently, we reported the isolation and characterization of a monokine, cachectin, that is made by macrophages stimulated with endotoxin (*3–6*). Cachectin completely suppresses the synthesis of lipoprotein lipase (LPL) in adipocytes in vivo and in vitro (*3–6*). Further structural studies revealed a marked homology between cachectin and human tumor necrosis factor (TNF), and subsequent biological studies confirmed that purified cachectin had TNF activity (*7*).

Although most studies of cachectin/TNF have centered on its antitumor activity, the protein is produced, in vivo and in vitro, in response to LPS challenge (*6, 8–11*), and binds to high-affinity receptors present on a number of normal host tissues (for example, liver, muscle, and adipose tissue) (*3*). We have previously proposed that cachectin may function as a hormone to promote cellular responses which, in part, result in the mobilization of host energy reserves in response to invasion (*3, 8, 9*).

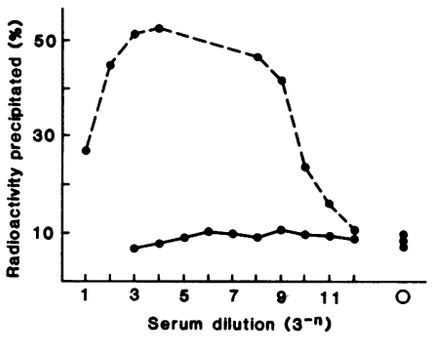
In the present study, we reasoned that cachectin/TNF might also play a role in the lethal metabolic effects of endotoxin-mediated shock. Accordingly, we passively immunized mice with antibody to cachectin/TNF and challenged them with lethal amounts of LPS. Cachectin/TNF was purified as previously described (*7*). The purified protein was prepared for use in immunization by electrophoresis in sodium dodecyl sulfate(SDS)–polyacrylamide slab gel. The gel was sliced after completion of electrophoresis, and approximately 5 μg of homogeneous protein (still contained within the gel slice) was emulsified in 1.0 ml of 0.05*M* ammonium acetate solution and 1.0 ml of Freund's complete adjuvant. A New Zealand White female rabbit was injected at multiple subcutaneous sites with this material. Four additional injections were given at monthly intervals, with 2 to 5 μg of cachectin/TNF, prepared as above but with Freund's incomplete adjuvant. Blood was withdrawn from the rabbit 4 days and 6 days after the final injection. Immune serum was tested for its ability to precipitate cachectin/TNF labeled with ^{125}I by the iodogen method (*12*) (Fig. 1). Approximately 50 percent of the tracer was precipitated when the serum dilution was 1:30,000; preimmune serum was nonreactive. The specificity of immune and preimmune sera was analyzed by immunoblotting (Fig. 2). A single major species, corresponding to murine cachectin/TNF, was labeled when blot transfers of medium from RAW 264.7 cells previously incubated with LPS were exposed to the immune serum. Occasionally, the presence of faint bands were noted in the gel above cachectin/TNF, possibly reflecting precursor molecules or glycosylation products. Preimmune serum showed no reactivity.

Neither immune nor preimmune serum contained antibodies reactive with LPS. This was assessed by the method of Neter *et al.* (*13*), in which human erythrocytes were passively sensitized with LPS and exposed to preimmune or immune serum over a range of dilutions between 1:2 and 1:1000. No agglutina-

tion was observed at any dilution, suggesting that if LPS antibodies were present, they were of extremely low titer. Similarly, no reactivity was observed when LPS (up to 50 μg) was subjected to electrophoresis, transferred to nitrocellulose, and exposed to immune serum.

When immune serum was administered to female BALB/c mice by intraperitoneal injection 1.5 hours before the intraperitoneal injection of 400 μg of LPS, a significant protective effect was demonstrable (Table 1) compared to the mortality rate observed among control mice treated with preimmune serum or with serum from other nonimmune rabbits. Mice pretreated with serum from rabbits immunized against bovine serum albumin according to a schedule similar to that used in cachectin immunization fared no better than the other control groups. After administration of the LPS, the mice in all of the groups appeared ill, and exhibited a febrile response. Thus, the immune serum did not abrogate the pyrogenic effect of LPS. A similar level of protection was observed when immunoglobulin G (IgG) prepared from the immune serum was administered to mice prior to the injection of LPS. In contrast, IgG prepared from nonimmune serum did not protect the mice.

The degree of protection provided by the immune serum was assessed by administering various doses of LPS to mice that had received either 50 μl of nonim-



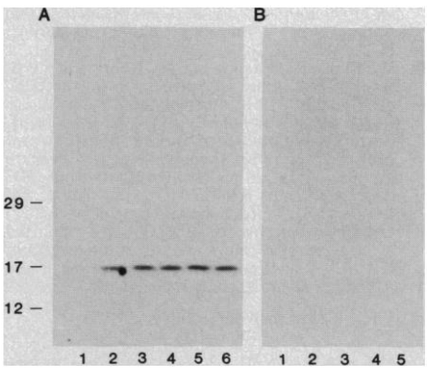
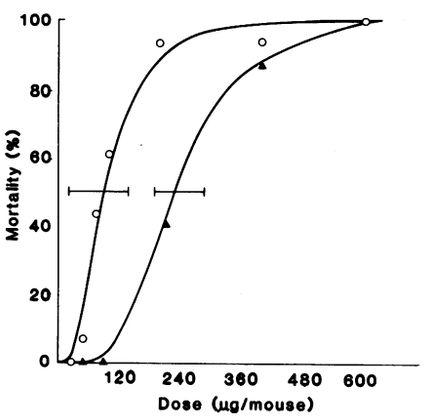


Fig. 1 (left). Immunoprecipitation of ^{125}I-labeled murine cachectin/TNF. Immune serum (dashed line) was prepared as described in the text. Preimmune serum (solid line) was obtained from the same animal. Immunoprecipitation reactions were carried out in conical polypropylene tubes. Serum samples were diluted to yield the final concentrations indicated, using Dulbecco's phosphate buffered saline containing 1 percent RIA-grade bovine serum albumin (Sigma) (PBS/BSA). Symbol: O, no serum added. Two microliters (approximately 0.2 ng; 1.4×10^4 count/min of labeled cachectin/TNF solution, prepared as described (*3*, *11*), was mixed with 20 μl of diluted serum. Solutions were incubated at 4°C for 5 hours. At that time, 10 μl of 1:5 suspension of washed *Staphylococcus aureus* bacterial adsorbant (Miles Laboratories) in distilled water was added to each tube. After 30 minutes, 1.0 ml of PBS/BSA was added to each tube. Samples were quickly mixed, and the immunoprecipitates were sedimented with a Beckman microfuge. Supernatants were aspirated, and the pellets counted for radioactivity with a Packard Autogamma Scintillation Spectrometer, model 578. Results are expressed as the percentage of radioactivity precipitated. Nonimmune sera from other rabbits (data not shown) were also incapable of precipitating labeled cachectin/TNF. Fig. 2 (right). Western blot of crude RAW 264.7 cell (mouse macrophage) conditioned medium, and of LPS. Lane 1 (A and B): 50 μg *Escherichia coli* strain 0127:B8 LPS (Difco). Lanes 2 to 6 (A) and 2 to 5 (B): 25 μl of LPS-stimulated RAW 264.7 cell conditioned medium (containing approximately 40 μg of total protein), prepared and concentrated 50-fold as described (*3*). Samples were applied to a 10 to 15 percent SDS-polyacrylamide gradient gel. After completion of electrophoresis, protein was transferred to nitrocellulose paper (Schleicher and Schuell) by using an electroblotting apparatus (Bio-Rad). Immune (A), and preimmune (B) sera were applied to the nitrocellulose blot at dilutions of 1:300, and reactive bands were identified with ^{125}I-labeled protein A (New England Nuclear) according to the protocol outlined by Hotez *et al.* (*14*). The band visible in (A) corresponds precisely in molecular weight to murine cachectin/TNF when the purified radioiodinated protein is subjected to electrophoresis and transferred in parallel as a marker. No reactive proteins were apparent when preimmune serum was applied to the blot.



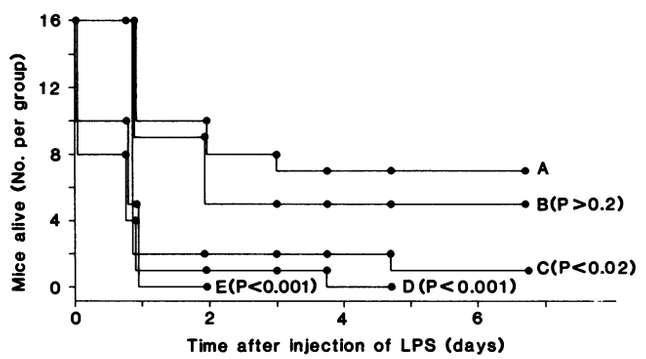


Fig. 3 (left). Estimation of the endotoxin LD_{50} in mice treated with immune and nonimmune sera. Male BALB/c mice (19 to 21 g) were randomly assigned to 11 groups, each containing 15 to 17 members. In four groups (triangles), mice were given intraperitoneal injections of 200 μl of immune serum diluted 1:4 with sterile isotonic saline; in the remaining seven groups (open circles), mice were given the same volume of 1:4 diluted nonimmune serum. After 5 hours, mice were given intraperitoneal injections of various quantities of *E. coli* strain 0127:B8 LPS, dissolved in sterile saline. All mice were observed for 48 hours after LPS injection. Curves were fitted to the data, and computation of LD_{50} was accomplished as described by Bliss (*15*), and by Litchfield *et al.* (*16*). Horizontal bars indicate the 95 percent confidence limits of LD_{50} determinations (91 μg for mice treated with nonimmune serum, and 227 μg for mice treated with immune serum). The administration of larger volumes of immune serum to mice (up to 200 μl) did not further improve their survival. Fig. 4 (right). Kaplan-Meier plot of survival after LPS treatment. Eighty male BALB/c mice (19 to 21 g) were randomly divided into five groups of 16 members each. Mice were protected by an intraperitoneal injection of 200 μl of antiserum to cachectin/TNF given 6 or 3 hours before, concurrent with, or 3 or 6 hours after intraperitoneal injection of 400 μg of *E. coli* strain 0127:B8 LPS (Difco). Censored determinations of survival were made at the indicated time points following LPS injection. (A) Survival was optimal when mice were treated with antiserum 6 hours before LPS administration. (B–D) A decrease in survival, with respect to these values, was noted when mice were given antiserum to cachectin/TNF 3 hours before LPS administration (B), at the time of LPS administration (C), 3 hours after LPS administration (D), or 6 hours after LPS administration (E). Tests of the null hypothesis that the time-related probability of survival in each group was equal to that in the group protected 6 hours before LPS administration were performed according to the Gehan method (*17*); *P* values (single-tail normal distribution; with Bonferroni correction for multiple comparisons) are indicated for each curve.

mune serum or 50 μl of immune serum 5 hours previously (Fig. 3). The median lethal dose (LD_{50}) of LPS in animals treated with immune serum was significantly higher than the LD_{50} for control mice treated with nonimmune serum.

The time at which the antiserum was administered relative to the time of LPS administration was found to be of crucial importance in producing a protective effect. Mice that were injected with immune serum 3 or 6 hours prior to administration of LPS fared better than those passively immunized at the time of LPS injection or several hours after (Fig. 4). This finding suggests that endotoxin elicits cachectin/TNF production soon after its administration, and that cachectin/TNF mediates its lethal injury within a very short time. In rabbits, cachectin/TNF is produced within minutes after the intravenous administration of LPS, and peak plasma concentrations are observed after 2 hours, with a rapid decline in concentrations occurring thereafter (*11*). Hence, in this model, the animal is exposed to high concentrations of the hormone only briefly; it is within this interval of time that effective antibody concentrations must be present if protection is to be achieved. Presumably the necessity for prior administration of the antiserum reflects the time required for complete distribution of the antibody within the recipient animal.

These data give evidence for the role of cachectin/TNF in mediating the lethal effects of LPS. Cachectin/TNF is clearly only one of the mediators responsible for the numerous pathological effects evoked by LPS, since the passively immunized mice become febrile, and continue to appear ill and distressed. It is possible, for example, that cachectin/TNF acts in concert with other mediators (for example, interleukin-1, interferons, and lymphotoxin) in order to elicit the lethal effect of LPS.

It is important to note that mice are relatively resistant to the effects of LPS when compared to most other mammals; rabbits, for example, are approximately 1000-fold more sensitive. In LPS-sensitive species, TNF may play a more prominent role as a mediator of shock. Immunization against TNF might then be expected to yield a higher level of protection.

The potential utility of passive immunization with antisera to cachectin/TNF in animals with shock induced by septicemia (or possibly other causes) needs further exploration. An obvious corollary is the possibility that agents which affect the synthesis or binding of cachectin/TNF to its receptor might be of utility in this setting without compromising the host's immune system. From these studies, a better understanding of the mechanisms by which the immune system influences other tissues may emerge.

Table 1. Protective effect of antiserum to cachectin/TNF. Female BALB/c mice (20 to 24 g) were randomly divided into six groups and injected intraperitoneally with serum from immune or nonimmune rabbits 1.5 hours before being injected with 400 μg of LPS from *E. coli* strain 0127:B8. Serum samples were diluted with sterile isotonic saline and injected in a final volume of 0.2 ml per mouse. LPS was also diluted in sterile saline and injected in a volume of 0.2 ml. Mortality was recorded daily, and the experiment was considered complete when no deaths were observed in any group for 3 days. The data show the number of survivors 7 days after LPS injection.

Serum	Serum volume injected (μl)		
	10	50	200
Immune	3/14	6/14	7/14
Nonimmune	0/14	1/14	0/14
P	> 0.05	< 0.05	<0.01

*Chi-square test.

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Morphological Novelty in the Limb Skeleton Accompanies Miniaturization in Salamanders

Abstract. *Salamanders of the genus* Thorius *(Plethodontidae) are among the smallest tetrapods. Hypotheses of limb skeletal evolution in these vertebrates were evaluated on the basis of estimates of natural variation, comparisons of skeletal homology, and analysis of molecular phylogeny. Nine carpal arrangements occur in* Thorius, *more than in all twelve related genera of typically larger salamanders; six of these arrangements are unique. They represent a trend toward a decrease in the number of separate cartilages that is independent of locomotor and ecological specialization. Miniaturization may be an important source of morphological novelty, distinct from local adaptation, in vertebrates.*

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The origin of novel morphological design is a primary focus of evolutionary morphology. One trend that may promote morphological novelty is phylogenetic decrease in body size, or miniaturization. Unique morphological arrangements are a common feature of dwarfed invertebrates (*1, 2*), many of which represent "entirely new types of organization" (*2*). In vertebrates, a frequent association between miniaturization and morphological novelty has been documented in many taxa, including teleost fishes (*3*), anuran (*4*) and urodele (*5–7*) amphibians, and squamate (*8–10*) and amphisbaenid (*11*) reptiles. Size decrease also has been implicated as a critical factor in the evolution of higher taxa such as frogs and salamanders (*5*), lizards (*8*), and snakes (*10*).

Lungless salamanders (Plethodontidae) provide some of the best examples of miniaturization among vertebrates. Decreased body size has evolved in several lineages; one, the Mexican genus *Thorius*, comprises the smallest extant tailed tetrapods (*6, 7, 12*). In this report I analyze the consequences of miniaturization in *Thorius* for forelimb skeletal morphology. The analysis is based on quantitative estimates of natural variation in limb osteology, on a comparison of skeletal unit homology, and on an electrophoretically derived molecular phylogeny. It reveals the following: (i) miniaturization of the genus as a whole correlates with the appearance of several