1496, 9,475 ± 130; SI 1499, 9,580 ± 135; and SI 1498, 9,660 ± 150; and (iii) SI 1502, 10,240 ± 110. The pooled mean age for the second group is 9,560 ± 80 years. Oxford dates on charcoal from units 26 and 62 are also compatible at the 5 percent level. If the Geochron dates are introduced, without regard to the stratigraphy and with all dates segregated to homogeneity at the 5 percent level, four discrete occupations may have occurred earlier than 9,000 years ago. The first and the fourth would be represented by single determinations of 12,560 ± 360 years (GX 1859) and 9,140 ± 90 years (SI 1497). The second occupation would be represented by the pooled mean age of two Geochron assays on charcoal (10,510 ± 210 years), one Smithsonian charcoal age (10,240 ± 110 years), and the pooled mean age of six Oxford assays on cordage and wood artifacts (10,100 ± 65 years). A third occupation would be represented by a Geochron date of 9,790 ± 240 years ago), a pooled mean of three Smithsonian dates (9,560 ± 80 years ago), and the pooled mean of seven Oxford dates (9,425 ± 55 years ago), all on charcoal. As judged from the Oxford dates, the four discrete occupations would have occurred about 12,200, 10,100, 9,400, and 9,000 years ago. Andean artifact typology is not exact enough to support this proposal.

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A Macrophage Factor Inhibits Adipocyte Gene Expression: An in Vitro Model of Cachexia

Abstract. *Certain infections and malignancies in mammals cause the development of a condition known as cachexia in which the animal continues to lose weight, often while consuming an adequate diet. When macrophages are stimulated with an endotoxin, they produce a factor or factors, termed cachectin, that inhibits the activity of fat-producing (lipogenic) enzymes in cultured adipocytes. This effect may reflect one of the physiological bases for cachexia. In the present study, clones of complementary DNA from genes whose expression is increased during the differentiation of adipocytes were used to study the molecular basis of cachectin's actions. In the presence of cachectin, the expression of the corresponding genes was reversibly and specifically inhibited. Furthermore, when mature adipocytes were exposed to cachectin, the messenger RNA's of those genes diminished and rapidly approached the levels present before differentiation.*

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The development of a chronic catabolic state is a hallmark of certain infections and malignancies. The weight loss that accompanies this condition is termed cachexia, and is associated with the mobilization of triglycerides from adipose tissue, a process that often persists in spite of adequate caloric intake. A factor or factors (termed cachectin) produced by endotoxin-stimulated macrophages in vitro dramatically decreases the synthesis and activity of key lipogenic enzymes of cultured 3T3L1 adipocytes (*1*, *2*). In this study, we used the stable adipogenic cell line TA1 to examine the mechanism of inhibition of lipogenic enzyme activity.

When cultured in monolayers, TA1 adipocytes develop a typical adipocyte morphology approximately 3 days after reaching confluence (*1*). As this differentiation occurs, several genes are expressed whose activity is evident only when differentiation has been initiated (*3*) (clones 1, 20, 28, 47). These messenger RNA's (mRNA's) are expressed largely or completely as a result of transcriptional activation of the corresponding genes (*4*). To assess the influence of cachectin on the coordinate induction of these adipose genes, we added cachectin to preconfluent TA1 cells and to TA1 cells on the day they reached confluence. Total RNA isolated from these and control cells 6 days after they reached confluence was probed with radiolabeled cDNA's (complementary DNA's) of genes whose expression was observed in adipocytes, but not in preadipocytes. Treatment with cachectin prevented the accumulation of adipose-inducible mRNA's (Fig. 1). Lipid accumulation was also completely inhibited by cachectin. Cultures of TA1 cells treated with cachectin were maintained for as long as 23 days without the appearance of neutral lipid, as detected by staining with oil red O. However, on removal of cachectin from the media, adipocyte morphology returned as did the expression of adipose inducible genes (for example, see clone 1 in Fig. 2).

The effects of cachectin are not due to endotoxin itself, since control supernatants from RAW 264 cells to which endotoxin has been added do not inhibit lipid accumulation or the production of lipogenic enzymes (*1*, *2*). Furthermore, cachectin does not generally affect gene expression; for example, the level of β-actin mRNA is unaffected by cachectin (Fig. 1). In addition, cachectin treatment of preadipocyte cultures does not affect cell growth or viability. In experiments similar in design to those in Fig. 1, cellular proliferation was determined by cell counting and [^{3}H]thymidine incorporation. Control and cachectin-treated

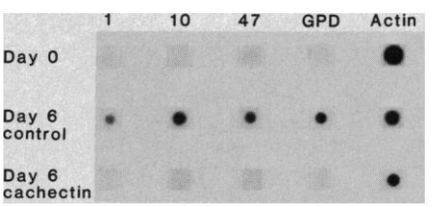
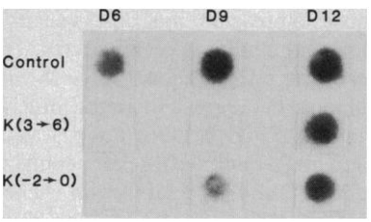


Fig. 1. TA1 cells, a stable adipogenic cell line derived from 5-azacytidine treatment of 10T1/2C18 cells (*3*, *10*, *11*), were grown in Eagle's basal medium supplemented with 10 percent heat-inactivated fetal calf serum. Dexamethasone ($10^{-6}M$) was present in the medium for the first 3 days after the cells reached confluence, and bovine insulin (5 μg/ml) for the first 6 days after confluence. Conditioned medium from the macrophage cell line RAW 264 treated with endotoxin (24 hours at 10 μg/ml in serum-free medium) was first added to preadipocyte cultures 2 days before they reached confluence at a concentration of 10 μl/ml, which inhibits 90 percent of lipoprotein lipase activity in cultured adipocytes. Cell cultures were resupplemented with hormones at day 0 (confluence) and day 3. Cells were harvested at day 6. Total RNA was isolated by the method of Chirgwin *et al.* (*12*), and applied to nitrocellulose in a dot blot apparatus (BRL). Nick-translated cDNA clones of genes whose expression is seen only in differentiated TA1 adipocytes (clones 1, 10, and 47, and glycerophosphate dehydrogenase) (*3*), as well as a β-actin cDNA clone were used to probe these filters under hybridization conditions previously described (*1*). Filters were washed, then exposed to XAR5 film at −70° with an intensifying screen.

cultures were indistinguishable by both methods at confluence. Cell viability, as determined by trypan blue exclusion and a clonal growth assay (*5*), was also equivalent in control and cachectin treated cells.

To determine whether the cachectin-mediated inhibition of adipose-specific mRNA accumulation is transcriptionally regulated, we used nuclear transcription assays. The normal developmental increase in gene transcription was inhibited by cachectin (see clones 1 and 28 in Fig. 3). Similar results were obtained for cDNA's for glycerophosphate dehydrogenase (GPD) and clone 47. However, the effect of cachectin in reducing transcriptional activity appeared to be selective: the transcription rates of β-actin in preconfluent cells, differentiated adipocytes, and cells treated with cachectin during differentiation were equivalent (Fig. 3).

In adult mammals, adipocytes undergo little or no proliferation (*6*). Thus the effect of cachectin on preconfluent TA1 cells in culture, although useful in investigating the coordinate regulation of adipose genes in development, probably does not provide a realistic model of mammalian cachexia. To model the in vivo situation more closely, we used mature adipocyte cultures to which we added cachectin. After 4 to 6 days of exposure to cachectin, most cells lost their neutral lipid. In typical experiments, 70 to 80 percent of cells would become laden with large lipid droplets when cachectin was first added; 6 days later, approximately 10 percent of cells would have identifiable triglycerides when stained with oil red O. Alterations in adipose-specific RNA's occurred more rapidly than lipid mobilization (Fig. 4). By 12 to 24 hours after the addition of cachectin to mature TA1 adipocytes, there was a greater than 90 percent decrease in the levels of such RNA's. We also found that the decrease in GPD mRNA level after cachectin addition paralleled decreases in GPD enzyme activity.



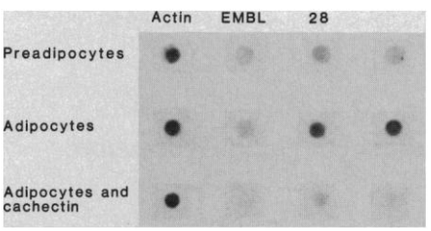


Fig. 2 (left). Dot blots were performed as described in Fig. 1. Cachectin (K) was present in the medium at the times indicated. RNA's were analyzed with clone 1 cDNA at 6, 9, and 12 days (D) after confluence. Fig. 3 (right). Transcription assays were performed using the method described by Vannice *et al.* (*13*) and Israel and Whitlock (*14*), as modified by Chapman *et al.* (*4*) for adipose cells. Cultured cells were chilled to 4°C, media aspirated and washed with phosphate-buffered saline. Hypotonic buffer (1 ml of 20 m*M* tris-HCl, *p*H 8.0, containing 4 m*M* $MgCl_2$, 5 m*M* $CaCl_2$, 0.5 m*M* dithiothreitol) was added to plates. After 5 minutes, 1 ml of lysis buffer (0.6*M* sucrose, 0.2 percent NP-40, and 0.5 m*M* dithiothreitol) was added, and cells were scraped from the tissue cultures dishes. After Dounce homogenization, nuclei were pelleted at 500*g*, washed once in resuspension buffer (0.25*M* sucrose, 10 m*M* tris-HCl, *p*H 8.0, 10 m*M* MgCl, 1 m*M* dithiothreitol) repelleted, then resuspended in 50 m*M* Hepes, *p*H 8.0, 90 m*M* NH_4Cl, 5 m*M* MgCl, 0.5 m*M* $MnCl_2$, 2 m*M* dithiothreitol, 0.1 m*M* EDTA, 0.4 m*M* each of adenosine triphosphate, cytosine triphosphate and guanosine triphosphate, 10 percent glycerol, and bovine serum albumin (10 μg/ml). Nuclei were incubated with α^{32}P-uridine triphosphate (600 Ci/mmol; ICN) at a concentration of 2 mCi/ml for 40 minutes at 25°C with gentle shaking. RNA was harvested from nuclei as described by Smith *et al.* (*15*) and modified by Chapman *et al.* (*4*), and hybridized to linearized cDNA's that had been applied to nitrocellulose filters and baked for 2 hours at 80°C in a vacuum oven. Filter prehybridization and hybridization conditions were those of Friedman *et al.* (*16*). Hybridizations were performed for 3 days at 42°C with approximately 15×10^6 cpm per reaction mixture applied in 150 μl volume to dots of adipose cDNA's for clones 1 and 28, as well as β-actin and pEMBL plasmid controls.

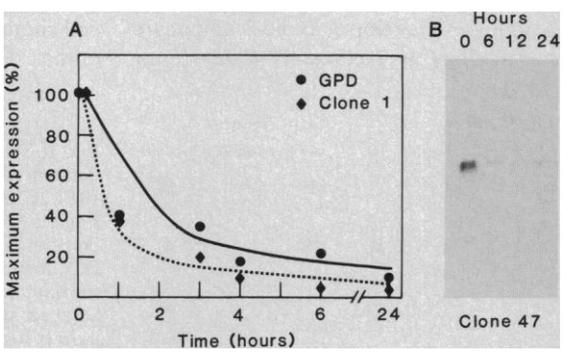


Fig. 4. (A) Cachectin (10 μl/ml) was added to day 6 adipocyte cultures differentiated as described in Fig. 1. Total RNA was isolated from cells at the indicated times after cachectin exposure, and applied to nitrocellulose with a slot-blot apparatus (Schleicher and Schuell). Filters were probed with the indicated cDNA's, washed, autoradiographed, and scanned by using a Hoeffer GS300 densitometer attached to a reporting integrator (Hewlett-Packard). Points shown were normalized for differences in amount of applied RNA with the use of a cDNA probe made to total cellular RNA. The levels of β-actin mRNA were also determined and found not to be altered by treatment of cells with cachectin. (B) Approximately 12 μg of total RNA was brought to a final concentration of 2.2*M* formaldehyde, 30 percent formamide, 10 m*M* NaH_2PO_4, *p*H 7.0, and heated for 15 minutes at 56°C. Samples were subjected to electrophoresis in a 1.0 percent agarose formaldehyde gel with the final concentration of 2.2*M* formaldehyde, 20 m*M* MOPS, *p*H 7.0, 5.0 m*M* sodium acetate and 1 m*M* EDTA (*17*). Gels were washed in distilled water for 3 minutes, washed twice for 30 minutes in 10 m*M* $NaPO_4$ (*p*H 7.4) and 1 m*M* EDTA, then transferred to nitrocellulose. The inset shows a representative filter probed with clone 47 DNA.

When cachectin is added to the medium of adipocytes in culture, the major lipogenic enzymes are diminished within hours. The rapid inhibition of adipose-specific mRNA's in response to cachectin has not been described previously as a mechanism by which mammalian tissues respond to injury. In mammals, there is a close physical association in

The similarity of this cultured adipocyte model to physiological events in the whole organism is striking. The macrophage is one of the primary effector cells by which the body reacts to foreign stimuli. Many of the initial host responses to infection and other injury are mediated by macrophage factors. Thus, the cachexia associated with chronic infections and malignancies may represent a persistence of what is initially a physiologic response to injury. The exact nature of the macrophage factor (or factors) responsible for this response is still undefined. The experiments described here were carried out with crude conditioned media of endotoxin-stimulated macrophages. A purified macrophage factor, interleukin-1 (mouse recombinant IL-1), does not inhibit adipocyte differentiation. However, Beutler *et al.* (*7*) have recently purified to homogeneity a protein whose effects on lipoprotein lipase activity and synthesis are similar to those of cachectin. This protein has a high degree of sequence homology to human tumor necrosis factor (TNF), another macrophage secretory product (*8*). Experiments with recombinant human TNF show that the morphologic effects of this substance on adipocyte differentiation and lipid mobilization are similar to those of cachectin (*9*).

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-