PURIFICATION OF CACHECTIN, A LIPOPROTEIN LIPASE-SUPPRESSING HORMONE SECRETED BY ENDOTOXIN-INDUCED RAW 264.7 CELLS

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In the course of recent studies aimed at elucidating the biochemical mechanism of cachexia in rabbits infected with *Trypanosoma brucei*, it was noted (1) that animals with a minimal parasite burden became moribund and exhibited an extreme hypertriglyceridemia, with a marked elevation of plasma very low density lipoprotein (VLDL).[1] The hypertriglyceridemic state was remarkable in view of the severe wasting diathesis that accompanied this experimental infection. The elevation of plasma VLDL was shown to result from a clearing defect, caused by a loss of peripheral tissue lipoprotein lipase (LPL) activity.

A similar deficiency of LPL activity was noted (2) in C3H/HeN mice after administration of *Escherichia coli* lipopolysaccharide (LPS). In contrast, the loss of LPL activity was not demonstrable in C3H/HeJ mice, which are genetically resistant to LPS. This resistance to endotoxin-induced LPL deficiency could be circumvented by the administration of serum obtained from endotoxin-sensitive animals that had been injected with LPS 2 h previously. Similarly (2, 3), resistance could be overcome by injecting conditioned medium from endotoxin-stimulated thioglycollate-elicited peritoneal macrophages, obtained from sensitive mice.

Macrophages and macrophage cell lines (i.e., RAW 264.7) were subsequently shown to elaborate, in response to endotoxin (4) or other bacterial and protozoal products (5), a monokine that could suppress the activity of LPL in the adipocyte cell line 3T3-L1. The mechanism of LPL suppression appears to involve the selective inhibition of LPL biosynthesis.

In the present paper, we report the isolation of cachectin, the macrophage factor that suppresses LPL activity in 3T3-L1 cells. The purified monokine is a

This work was supported in part by grant 1 K11 AM01314 01 from the National Institutes of Health and grant 84077 from the Rockefeller Foundation. Address correspondence to B. Beutler. Present address of J. Mahoney is: Department of Surgery, University of Minnesota Medical School, 516 Delaware Street S.E., Minneapolis, MN 55455.

[1] *Abbreviations used in this paper:* Con A, concanavalin A; DME, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; IL-1, -2, interleukin 1 and 2; LAF, leukocyte-activating factor; LPL, lipoprotein lipase; LPS, lipopolysaccharide; PBS, phosphate-buffered saline; PTS, percent total suppression; RBC, erythrocytes; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TNF, tumor necrosis factor; VLDL, very low density lipopolysaccharide.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/85/05/0984/12 $1.00
Volume 161 May 1985 984–995

17 kilodalton protein that binds to adipocytes and several other cells types, by means of specific high affinity receptors.

Materials and Methods

3T3-L1 Cell Culture and Bioassay. 3T3-L1 cells (6–8) were plated in 24-well Linbro plates (Flow Laboratories, Inc., McLean, VA) at a density of 100,000 cells per well in Dulbecco's modified Eagle's medium (DME) (Gibco Laboratories, Grand Island, NY) supplemented with 10% calf serum, and maintained at 37°C in a 10% CO_2 environment. Medium was changed every other day; after 1 wk, cells were differentiated by incubation for 48 h in medium supplemented with 10% fetal bovine serum (FBS), 10 μg/ml bovine insulin, 0.5 mM methylisobutylxanthine, and 1.0 μM dexamethasone. Differentiated cells were maintained in medium containing 10% FBS and 50 ng/ml bovine insulin (changed every other day) until their use for bioassay or cachectin-binding studies 1 wk later.

Samples to be assayed, in volumes ≤0.1 ml, were applied to individual wells of differentiated 3T3-L1 cells, each containing 1.0 ml of medium, and placed in a 10% CO_2 incubator at 37°C for 12–18 h. At the end of this incubation, the medium was aspirated and replaced with 0.5 ml of DME containing 10% FBS, 50 ng/ml insulin, and 10 U/ml heparin. After 1 h at 37°C, the heparin-releasable LPL activity was measured according to the method of Nilsson-Ehle and Schotz (9).

Percent of total LPL suppression (PTS) in a sample with LPL activity x was defined as follows: $PTS = (C - x/C - m) \times 100$, where C is the LPL activity produced by wells of 3T3-L1 cells that have not been exposed to cachectin, and m is the LPL activity of heparinized medium alone. To avoid day-to-day assay variability, the unknown sample results were compared with a standard preparation of cachectin included in all bioassays. This standard preparation of crude cachectin was aliquoted into small tubes and stored at −80°C. 1 U of bioactivity was defined as the amount of cachectin yielding a PTS equivalent to that achieved by addition of 1.0 μl of the standard to the 1.0-ml assay system. Use of a standard solution of cachectin also assured a linear estimate of the amount of bioactivity present in unknown samples.

Cachectin Production. RAW 264.7 cells (10, 11) were grown to confluence in RPMI 1640 medium (Gibco Laboratories) supplemented with 10% FBS, using 150-cm^2 T flasks maintained at 37°C in a 5% CO_2 environment. Before induction of cachectin secretion, RAW 264.7 cells were washed extensively with Hanks' balanced salt solution (Gibco Laboratories) buffered with 25 mM Hepes, pH 7.4 (Research Organics, Inc., Cleveland, OH) to remove contaminating serum proteins. Each flask was stimulated with 100 ml of RPMI 1640 medium containing 1 μg/ml of *E coli* LPS (Difco Laboratories, Inc., Detroit, MI) and 50 mM Hepes, pH 7.4. After 22 h of incubation at 37°C, the medium was filtered through a 0.22 μm filter and frozen at −20°C.

Purification of Cachectin. 855 ml of stored frozen medium was thawed and concentrated under 50 psi of nitrogen at 4°C using a stirred cell with a PM-10 filter (Amicon Corp., Danvers, MA). After concentration to 2% of the initial volume, the retained protein solution was repeatedly diluted with distilled water and reconcentrated to desalt the sample before isoelectric focusing.

Isoelectric focusing was performed at 4°C in a water-cooled column with a 400 ml capacity. A glycerol gradient was prepared in this column with the protein sample layered in the center as follows: a linear gradient of glycerol from 40 to 21% (vol/vol) was added; the sample was supplied with a peristaltic pump; and a glycerol gradient of 19 to 0% (vol/vol) was layered above the sample. Both upper and lower segments of the gradient contained Servalyte, pH 3–10 (Serva Fine Biochemicals, Inc., Garden City Park, NY), diluted 1:16 with water. The sample was prepared by mixing the salt-free concentrate (55 ml vol) with 4.3 ml Servalyte, pH 3–10, and 16.7 g glycerol. The anode solution (at the bottom of the column) was 0.01 M imidodiacetic acid in 40% glycerol, and the cathode solution (at the top of the column) was 0.01 M ethylenediamine in H_2O. The separation was carried out at an initial current of 18 mA and an initial voltage of 630 V. The voltage was increased as current declined over the course of the run, but power output was not allowed to exceed 16 W.

After 24 h, the column was drained under gravity into 150-drop fractions. Precipitated proteins were removed by centrifugation at 3,000 *g* for 20 min at 4°C. A drop of phenol red solution was added to each decanted supernatant, and acidic samples were neutralized by addition of saturated tribasic sodium phosphate solution, while basic samples were neutralized by addition of 30% phosphoric acid solution. Cachectin bioactivity was determined by assaying 2 μl from each fraction. Six fractions, corresponding to the peak of bioactivity, were pooled for further purification.

The pooled peak fractions were dialyzed at 4°C against several changes of Dulbecco's phosphate-buffered saline (PBS) (Gibco Laboratories) to remove ampholytes and glycerol. The dialyzed material (94 ml in volume) was passed over a concanavalin A (Con A)-Sepharose column (Pharmacia Fine Chemicals, Uppsala, Sweden) with a bed volume of 2.0 ml for 1 h at 4°C. An additional 10 ml of PBS was then run through the resin and pooled with the rest of the filtrate. The filtrate was dialyzed against two changes of distilled water at 4°C and lyophilized.

The sample was redissolved in 2 ml of a solution containing 30 mM Tris-HCl buffer, pH 6.8, 5% glycerol, 1 mM β-mercaptoethanol, and 0.002% bromphenol blue, and then subjected to electrophoresis under nondenaturing conditions. The sample was applied across the stacking gel of a 1.5 mm × 14.2 cm × 13.6 cm slab gel with a 6–13% linear polyacrylamide gradient. A Tris-glycine buffer system was used as follows: the cathode buffer was 38 mM Tris base and 40 mM glycine; the anode buffer was 63 mM Tris base and 50 mM HCl; the stacking gel buffer was 0.125 M Tris-HCl, pH 6.8; and the separating gel buffer was 0.375 M Tris-HCl, pH 8.8 (final concentrations). In addition to acrylamide (29.2%), the monomer solution contained 0.8% bis-acrylamide. To remove impurities, the monomer solution was stirred overnight with Amberlite MB-1 resin (Mallinckrodt, Inc., St. Louis, MO). The sample was electrophoresed at 4°C with a constant current of 20 mA until the bromphenol blue marker reached the bottom of the gel. After completion of electrophoresis, 1 cm of gel was removed from the lateral margins of the slab, since voltage artifacts caused nonhorizontal migration in these regions, and the remainder of the gel was sliced horizontally at 2-mm intervals. The protein was recovered from each slide by allowing it to diffuse into 2 ml of a 50 mM ammonium bicarbonate solution in a polypropylene tube over a period of 12 h at 4°C with gentle agitation. Electroelution was carried out in a tube gel apparatus against a 3,500-dalton-cutoff dialysis membrane, as described by Francis et al. (12). The fractions thus obtained were assayed for activity, and subjected to polyacrylamide gel electrophoresis in a denaturing system containing sodium dodecyl sulfate (SDS-PAGE) in 10–15% linear gradient gels under nonreducing and reducing conditions to assess purity. The protein bands were visualized by silver staining as previously described (13).

Analytical Column Chromatography. Gel filtration was carried out using fine grade Sephadex G-75 (Pharmacia Fine Chemicals) in a 7 mm × 1 m glass column, equilibrated with 0.1 M ammonium bicarbonate, pH 7.8. Enzyme grade urea (Bethesda Research Laboratories, Bethesda, MD) was added to the sample and the eluant buffer in experiments aimed at determining cachectin subunit size, and fractions were individually dialyzed against PBS before bioassay.

Studies With Radioiodinated Cachectin. Purified cachectin was radiolabeled by the iodogen method (14). The free iodide was removed by Sephadex G-25 gel filtration followed by extensive dialysis. Binding studies were carried out using 24-well Linbro plates containing recently differentiated 3T3-L1 cells (4×10^5 cells per 0.2 ml system). The solution used for binding studies consisted of DME medium, containing 10% FBS, 50 ng/ml insulin, and 50 mM Hepes buffer, pH 7.4. Varying quantities of labeled cachectin were added to this solution. Except where otherwise noted, the incubations were carried out on ice for 4 h, with slow agitation. The cell monolayers were then washed three times with 1.0 ml of medium, solubilized with 1.0 ml of 1 M NaOH, and counted in a gamma counter for a length of time sufficient to yield a standard error of <5%.

The muscle cell line C2 (the kind gift of Dr. Helen Blau of Stanford University Medical School, Palo Alto, CA) was also analyzed for cachectin binding. Cells were grown to confluence in 24-well Linbro plates. The plates were pretreated with calf skin collagen

(Calbiochem-Behring Corp., La Jolla, CA) by autoclaving 50 mg of collagen in 35 ml water and applying the sterile solution to each of the 24 wells per plate. The solution was then aspirated and the plates allowed to dry under ultraviolet light in a laminar flow hood. Growth medium used was DME supplemented with 20% FBS and 0.5% chick embryo extract (Gibco Laborories). Cultures were maintained at 37°C in a 5% CO_2 environment. The medium was changed daily, and cells were induced to form myotubules when they reached confluence by addition of DME containing 2% horse serum (Gibco Laboratories). After 48 h, the cells were used for binding studies. Measurement of cachectin binding to C2 myotubules was performed in a manner identical to that described for 3T3-L1 cells.

NCS female mice were obtained from the Laboratory Animal Research Center of The Rockefeller University. Mouse erythrocytes (RBC) were obtained by filtration of heparinized mouse blood over a microcrystalline cellulose column as previously described (15). Splenic lymphocytes were obtained by Ficoll-Hypaque (Pharmacia Fine Chemicals) sedimentation. Measurements of cachectin binding to erythrocytes and lymphocytes were undertaken with the use of the same procedures applied in the case of 3T3-L1 cells. However, incubation with labeled cachectin was performed in 1.5-ml Eppendorf tubes (Brinkmann Instruments, Inc., Westbury, NY). 4×10^7 mouse RBC and 1×10^7 mouse lymphocytes were used in each binding assay. Washing was accomplished by centrifuging three times at 4°C using a microfuge (Beckman Instruments, Inc., Fullerton, CA). The pellets of cells were counted directly.

Crude hepatocyte membranes were prepared by homogenization of 2 g mouse liver (wet weight) in 10 ml of 5 mM Hepes, pH 7.4. The homogenate was diluted to 50 ml with the same buffer and centrifuged at 200 *g* at 4°C for 5 min. The supernatant was divided into 2-ml aliquots and pelleted at 48,000 *g* in a 10 min spin. The resulting pellets were each suspended in 0.5 ml of DME supplemented with 10% FBS, to which labeled cachectin (1 ng/ml) was added, with or without unlabeled cachectin (300 ng/ml). Binding was allowed to occur for 4 h at 4°C. The membranes were then washed three times with fresh medium by centrifugation at 48,000 *g*. The pellets were counted for radioactivity.

Assays of Interleukin 1 (IL-1) Activity. These were carried out as previously described (16), using recombinant IL-1 (17) as a standard preparation.

Protein Determination. Estimates of protein concentration were made by means of a commercial Coomassie-based protein reagent (Bio-Rad Laboratories, Richmond, CA).

Results

Preparation of Cachectin from RAW 264.7 Cells and Characteristics in Crude Solution. ~10^5 U of cachectin bioactivity in 50 ml of medium could be obtained from a confluent flask containing 10^8 RAW 264.7 cells. The specific activity of the crude material was routinely observed to be ~4×10^5 U/mg protein. Very little additional cachectin could be obtained by longer incubation of the cells with LPS, or by restimulation with fresh medium containing LPS. On the contrary, a drop in specific activity was observed when such attempts were made.

No loss in bioactivity was observed over several weeks after storage of the crude preparation at neutral pH at −20°C, or over 5 d at 4°C. However, bioactivity was noted to be labile at acid pH, with a half-life of 24 h at 4°C at pH 5.0. Lability at low pH could be diminished by addition of 20% glycerol. For this reason, isoelectric focusing was carried out in glycerol rather than sucrose, and this step was performed as rapidly as possible.

Concentration and Isoelectric Focusing. Bioactivity was associated exclusively with the retentate when pressure dialysis was performed under the conditions described. Recovery after a 50-fold concentration was generally >90%, and was occasionally enhanced (Table I). The data in Table I are from a representative isolation; to date, the procedure outlined there has been followed 11 times.

TABLE I

Step	Volume	Total activity*	Total protein‡	Specific activity	Fold purification	Recovery
	ml	*U*	*mg*	*U/mg*		*%*
Unconcentrated crude	855	2.5×10^7	53.9	4.6×10^5	—	100
Concentrated crude	54	2.7×10^7	38.9	6.9×10^5	1.5	108
Isoelectric focusing	94	1.5×10^7	3.88	3.9×10^6	8.5	60
Con A–Sepharose	104	1.4×10^7	2.78	5.0×10^6	11	56
Nondenaturing PAGE	8	1.1×10^6	0.03	1.8×10^7	80	2

* Determined as described in text.
‡ Determined by protein assay (Bio-Rad Laboratories) except in the final step, when estimate was made by silver stain, with reference to a myoglobin standard.

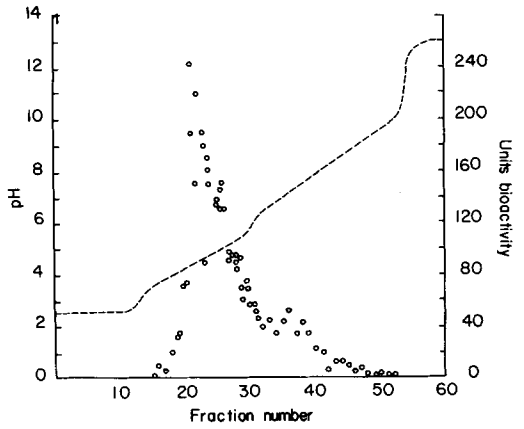

FIGURE 1. Preparative isoelectric focusing of cachectin. 150-drop fractions were obtained by draining the isoelectric focusing column under gravity as described in the text, and cachectin bioassays were performed on 2.0-μl aliquots taken from each fraction. Each open circle represents the result of a separate bioassay. Fractions in the region of the peak were assayed in duplicate or in triplicate. The units of bioactivity are described in Materials and Methods. The dashed curve indicates the pH gradient that was established.

Isoelectric focusing of the concentrated material revealed only one peak of bioactivity with an isoelectric point of 4.7 (Fig. 1). Ampholytes by themselves do not interfere with the bioassay. Recovery of bioactivity after isoelectric focusing was variable (10–60%) and depended to a large extent upon the total quantity of protein added to the column. The loss of bioactivity during isoelectric focusing appeared to result from coprecipitation of cachectin with other proteins present in the crude concentrate, since poor yields were associated with separations of large quantities of protein and the formation of large amounts of precipitate. Later experiments in which radiolabeled purified cachectin was added to the crude material as a tracer before isoelectric focusing supported this conclusion. Recovery was maximized by addition of a small sample to the column (50 mg total protein or less), the use of a glycerol gradient, and a short separation time (24 h or less), with immediate neutralization of samples after fractionation.

Con A–Sepharose Chromatography. Several protein species were removed by affinity chromatography on Con A–Sepharose with little or no loss of bioactivity.

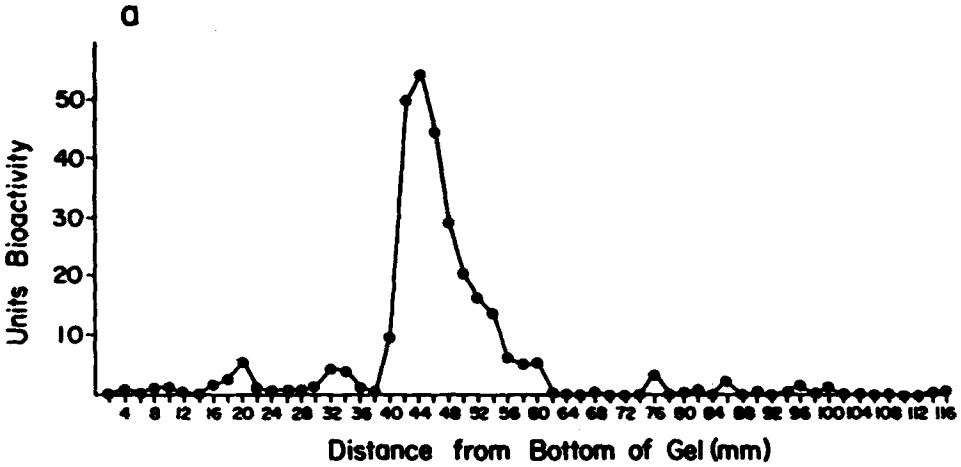

FIGURE 2. Preparative PAGE of cachectin. After isoelectric focusing and Con A–Sepharose chromatography, the sample was subjected to electrophoresis under nondenaturing conditions as described in the text. Serial 2-mm slices were cut from the gel and the bioactivity was eluted from each slice by allowing diffusion into ammonium bicarbonate solution. Bioassays were performed on 1-μl aliquots of each fraction. Units of bioactivity are plotted against the position at which each slice was made (relative to the bottom of the gel). Only one peak of activity is resolved, which corresponds to a single protein band visible upon staining of the nondenaturing gel.

Use of larger quantities of Con A–Sepharose resulted in lower recovery of cachectin activity, possibly because of hydrophobic interaction with the resin, since the activity could not be released by addition of 0.1 M α-methylmannoside.

PAGE. After PAGE under nondenaturing conditions, all of the bioactivity was found in one location on the gel, associated with a single protein band (Fig. 2, 3). In this step, only 10–20% of the bioactivity could be recovered from the sliced gel, either by electroelution or by allowing diffusion of the protein into dilute ammonium bicarbonate buffers. Longer diffusion times, repeated extraction, and the use of larger volumes of buffer had little effect. In our experience, recovery was maximized by the application of a highly concentrated sample to the gel, and by the use of purified acrylamide monomer in gel preparation.

SDS-PAGE Analysis of Electrophoretically Purified Cachectin. The apparent homogeneity of the isolated product was confirmed by electrophoresis in an SDS-PAGE system. The purified protein associated with cachectin activity, applied to SDS gels, yielded a single band of molecular weight 17,000 (Fig. 3). When microgram quantities of purified cachectin were electrophoresed in SDS gels, bioactivity could be recovered from unfixed slices by electroelution. The activity coincided with the presence of the 17,000 mol wt protein; no bioactivity was obtained from other regions of the gel (Fig. 4).

Molecular Weight of Purified Cachectin. When crude concentrates of cachectin were analyzed by means of Sephadex G-75 gel filtration, the bioactivity elution profile indicated a molecular weight in >70,000. A molecular weight estimate of 17,000 (dextran scale) was obtained, however, when crude concentrates were separated by gel filtration in the presence of 6 M urea, suggesting the formation of noncovalent multimers or aggregates with other proteins (data not shown).

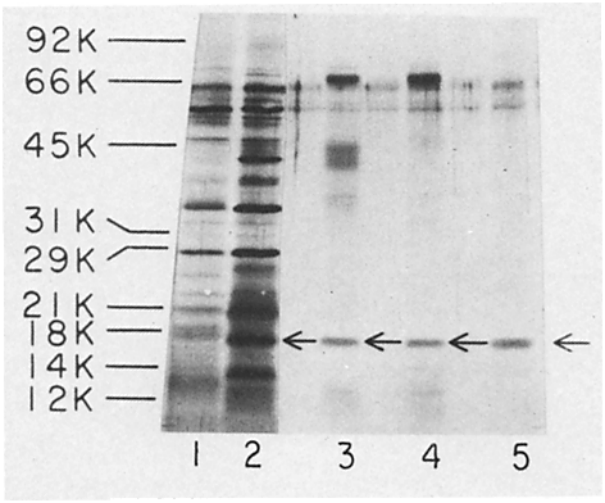

FIGURE 3. SDS-PAGE analysis of fractions from cachectin isolation. Samples of crude medium from endotoxin-induced and noninduced RAW 264.7 cell cultures, and purification intermediates were subjected to SDS-PAGE in a 10–15% linear polyacrylamide gradient gel. Samples were dialyzed against distilled water before electrophoresis. (*1*) 150 μl of noninduced RAW 264.7 cell medium; (*2*) 50 μl of induced RAW 264.7 cell medium; (*3*) 10 μl sample of pooled fractions from isoelectric focusing column; (*4*) 25 μl sample of Con A–Sepharose filtrate; (*5*) 80-μl sample of the peak fraction obtained by PAGE under nondenaturing conditions (homogeneous cachectin). Arrows indicate cachectin, which is entirely absent from the medium of noninduced cells, but is a major constituent of the medium of induced cells. Cachectin is successively enriched by the purification steps listed above. The high molecular weight bands visible in all lanes (including spacer lanes) are β-mercaptoethanol contaminants, and are not present in the samples themselves.

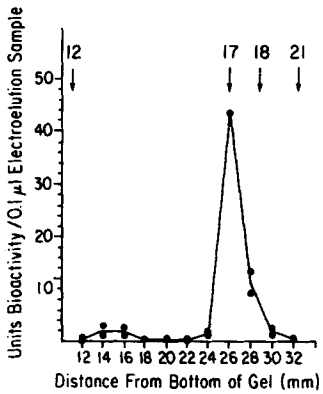

FIGURE 4. SDS-PAGE analysis of purified cachectin. A 20-μg sample of purified cachectin was boiled for 2 min in the presence of SDS and subjected to electrophoresis under denaturing conditions in a 10–15% linear polyacrylamide gradient gel. Serial 2-mm slices of this gel were electroeluted as described in the text. The electroelution fractions were diluted 1:100 with DME containing 10% FBS, and 10-μl aliquots were assayed for bioactivity. The peak of bioactivity corresponded precisely to the locations of the cachectin monomer. This was confirmed by reelectrophoresis of the eluates in a second SDS gel, followed by silver staining (data not shown). No bioactivity was found elsewhere in the gel. Arrows indicate the position of molecular weight standards.

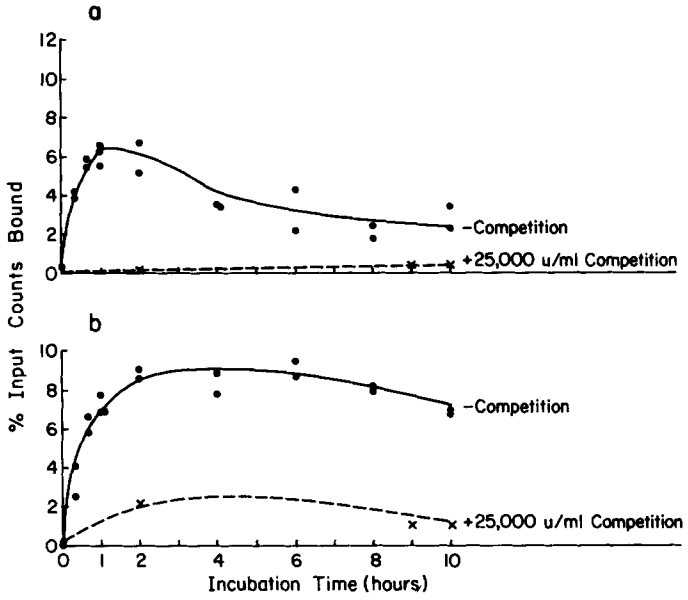

FIGURE 5. Binding of cachectin to 3T3-L1 cells. Individual monolayers of 3T3-L1 cells were exposed for varying lengths of time to radiolabeled, purified cachectin (40 U/ml bioactivity). Cachectin associated rapidly and specifically with intact 3T3-L1 cells at 37°C (*a*) and at 0°C (*b*) (solid lines). Nonspecific binding was determined at both temperatures by addition of an excess of unlabeled crude cachectin (25,000 U/ml; dotted lines). Data are expressed in terms of the percentage of input radioactivity binding to cell monolayers as a function of time. Duplicate or triplicate assays were performed at each time point. Details of the binding assay are provided in the text.

When purified cachectin was analyzed on SDS gels, both reduced and nonreduced samples exhibited an apparent molecular weight of 17,000. Gel filtration of purified cachectin on Sephadex G-75 columns provided an estimated molecular weight of 35,000, indicating the presence of noncovalent dimers (data not shown).

Radioiodination and Binding Studies. The purified hormone was radiolabeled with ^{125}I by the iodogen method. ~70% of the bioactivity was recovered, with an initial specific activity of $\sim 1 \times 10^6$ cpm/μg protein. The labeled material was subjected to SDS-PAGE, and measurement of sliced gels showed most of the radioactivity to be associated with the 17,000 mol wt protein. Attempts to label the protein using chloramine T resulted in a complete loss of bioactivity.

The purified, radioiodinated hormone was rapidly and specifically bound by cultured 3T3-L1 adipocytes at both 37 and 0°C (Fig. 5, *a* and *b*). Addition of 25,000 U/ml of unlabeled cachectin prevented binding by the labeled protein at all time points examined. Equilibrium was achieved within 2–3 h under the conditions described. A time-dependent decline in the quantity of label bound was observed at 37°C, possibly reflecting receptor-mediated endocytosis.

The characteristics of binding were exained by Scatchard analysis (Fig. 6*a*). The data suggest the existence of $\sim 10^4$ high-affinity sites per cell, and an association constant (K_a) of 3×10^9. Both differentiated 3T3-L1 cells and undifferentiated "pre-adipocytes" possessed receptors, with similar affinity and

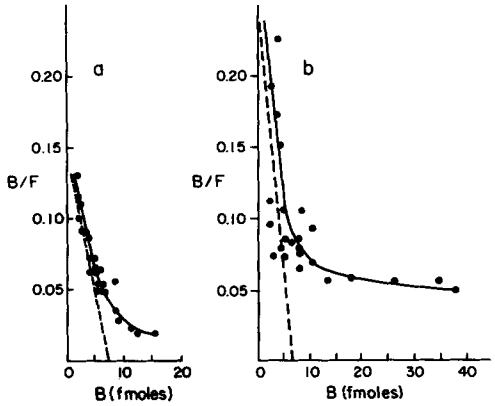

FIGURE 6. Scatchard analysis of ^{125}I-cachectin binding to intact differentiated 3T3-L1 cells and C2 myotubules. Individual monolayers of 3T3-L1 cells (*a*) or C2 myotubules (*b*) were exposed to increasing concentrations of radiolabeled cachectin, 0.5–30 ng/ml. Each point represents a measurement of cachectin binding to a cell monolayer at a concentration in the range noted above. *B*, counts bound; *F*, counts not bound (calculated by subtracting *B* from input. Calculation of molar concentration assumes a molecular weight of 17,000, and a reaction volume of 0.2 ml. 4×10^5 3T3-L1 cells or C2 cells were present per monolayer.

density. The muscle cell line C2 also possessed a specific cachectin receptor. Again, $\sim 10^4$ high-affinity sites per cell and a K_a of 3×10^9 were observed (Fig. 6*b*). Mouse liver membrane preparations also exhibited specific cachectin binding (data not shown). On the other hand, erythrocytes and lymphocytes lacked detectable quantities of cachectin receptor (<200 copies per cell).

Assays for IL-1 Activity. In view of its similar size, tissue of origin, and endotoxin inducibility, it appeared possible that the protein isolated was identical to IL-1. The large quantity of cachectin produced by RAW 264.7 cells suggested that this was not the case, particularly since it was found that the cells produced very little IL-1, as measured by leukocyte-activating factor (LAF) activity under the conditions used to elicit cachectin. Purified cachectin preparations lacked LAF activity when assayed over a wide range of concentrations up to 1 nM, under conditions at which recombinant IL-1 could be detected at 10^{-11} M. Moreover, micromolar concentrations of highly purified recombinant IL-1 failed to compete with the radioiodinated cachectin for receptor binding. It therefore appears that cachectin is distinct from IL-1.

Discussion

In the present paper, we describe the isolation of a unique macrophage protein that is responsible for suppression of LPL activity in 3T3-L1 adipocytes. The purified protein has a monomeric molecular weight of 17,000 on SDS gels, and an isoelectric point of 4.7. Under nondenaturing conditions, purified cachectin apparently exists as a dimer.

1 U of bioactivity corresponds to $\sim 2 \times 10^{-15}$ mol of isolated monomer. Thus, a 2 pM solution of cachectin is readily detectable by means of the bioassay described. This estimate of specific activity must be considered minimal, since an unknown quantity of the protein may have been rendered biologically inactive during purification.

The isolation of cachectin was facilitated by the use of the murine macrophage cell line RAW 264.7, which allowed large-scale production of the protein for analysis and purification. In addition to being a homogeneous population, the cells could be maintained in serum-free medium during endotoxin stimulation. The RAW 264.7 cell line was generated through transformation of mouse peritoneal cells by Abelson leukemia virus (10, 11). The cells resemble macrophages morphologically and functionally. They possess receptors for complement and Fc fragments of IgG, and are able to phagocytose neutral red, opsonized RBC, and latex beads. We have previously demonstrated (4) marked similarity between the cachectin activity produced by RAW 264.7 cells and that produced by thioglycollate-elicited peritoneal macrophages. Further work is needed to prove actual identity.

SDS-PAGE analysis of culture medium derived from LPS-stimulated and unstimulated RAW 264.7 cells revealed that cachectin is one of the major endotoxin-inducible secretory proteins. It constitutes 1–5% of the total protein secreted by stimulated RAW 264.7 cells, and is clearly visible as a distinct 17,000 mol wt band on SDS gels. Medium from unstimulated cells completely lacks cachectin bioactivity and the 17,000 mol wt band. It would appear, based on estimates of the specific activity of purified cachectin, that the 17,000 mol wt protein accounts for most, if not all, of the bioactivity present in conditioned cell medium.

The possible relationship of cachectin to other monokines (e.g., interferons, glucocorticoid-antagonizing factor, tumor necrosis factor (TNF), or other bioactivities produced by macrophages) has not been fully assessed. Purified IL-2 and crude TNF preparations appear to lack LPL-suppressing activity (our unpublished observation). From the bioactivity data presented above, it is clear that cachectin is distinct from IL-1, despite similar molecular weights and isoelectric points.

Using labeled cachectin, we have demonstrated the existence of specific receptors for cachectin on differentiated 3T3-L1 cells and preadipocytes, C2 muscle cells, and mouse liver cell membranes. On the other hand, receptors were not detected on mouse-erythrocytes or lymphocytes. The receptor density on 3T3-L1 cells and on C2 cells was $\sim 10^4$ per cell. The K_a in both cases was determined to be 3×10^9. From the value of the K_a, the degree of receptor occupancy can be determined as a function of cachectin concentration. It would appear that occupancy of as few as 5% of the receptors present on a 3T3-L1 cell (achieved at 2×10^{-11} M) is sufficient to elicit 70–80% suppression of LPL. This type of relationship has been noted with many other hormone receptor–binding systems.

Although the macrophage is not a characteristic endocrine cell, it can produce a monokine, cachectin, which may be described as a hormone in the classical sense, since it is produced by a specific group of cells, and influence, via a high affinity receptor, the behavior of other cell types. As such, it provides an example of the endocrine capabilities of reticuloendothelial cells. In view of the fact that muscle, liver, and adipose tissues possess receptors for cachectin, a wide range of biological responses is anticipated.

We hypothesize that cachectin may serve to mobilize the energy reserves of a mammal confronted by acute infection, to meet increased metabolic demands.

When chronically evoked, as in parasitic infections, cachectin may contribute to the protein and lipid catabolism that ultimately reduce the host to a state of cachexia. Further inquiry into this potential role of cachectin will be facilitated by the availability of pure material, and by the use of the receptor as the basis of a highly specific assay.

Summary

Previous studies have indicated that endotoxin and other bacterial and protozoal products can stimulate macrophages to produce a factor that can suppress the activity of the enzyme lipoprotein lipase (LPL), in vivo and in vitro. In the present report we describe the purification of this factor, cachectin, to apparent homogeneity from the conditioned medium of endotoxin-stimulated RAW 264.7 cells. The isolated protein has an isoelectric point of 4.7 and a subunit molecular weight of 17,000. Although cachectin's isoelectric point and molecular weight are similar to those described for interleukin 1, pure cachectin has no leukocyte-activating factor (LAF) activity.

Cachectin at a concentration of 10^{-11} M has the ability to suppress the LPL activity of the 3T3-L1 adipocyte cell line by 80%. Binding studies using radiolabeled cachectin and 3T3-L1 adipocytes and C2 myotubules revealed ~10^4 high-affinity receptors per cell on both cell types (K_a, 3×10^9). Cachectin receptors were also present on liver membranes but were absent on erythrocytes and lymphocytes.

The isolation of cachectin and characterization of its receptor should facilitate further investigations into the role of cachectin and other macrophage mediators in the metabolic derangements that occur during infection and cachexia.

We are indebted to Dr. Peter Lomedico of Hoffmann-La Roche, Inc. for providing the recombinant IL-1 used in our work, and to Dr. Angela Piperno of The Rockefeller University for her assistance in performing IL-1 assays. Dr. Elizabeth Carswell kindly provided us with tumor necrosis factor, and Dr. Edward Reich supplied IL-2, both of which were tested for LPL-suppressing activity. We are also indebted to Mrs. Madeline Velez-Phillips and to Mr. David Greenwald for their able technical assistance.

Received for publication 4 February 1985.

Note added in proof: We have recently obtained N-terminal sequence data for cachectin, and noted strong homology to the sequence for human TNF. We are currently investigating the relationship between these two molecules.

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