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Effect of endotoxin-induced monokines on glucose metabolism in the muscle cell line L6

(cachectin/tumor necrosis factor/RAW 264.7 cells/glycolysis/glycogenolysis/glucose transporter)

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ABSTRACT **Exposure of fully differentiated L6 myotubes to a crude monokine preparation from endotoxin-stimulated RAW 264.7 cells resulted in a rapid and substantial (70%) increase in fructose 2,6-bisphosphate concentration coincident with a depletion of cellular glycogen and an increased lactate production. During the time required for glycogen depletion (3 hr), stimulation of 3-*O*-methyl-D-glucose and 2-deoxy-D-glucose uptake was initiated and observed to reach a maximum enhancement of 200% 12–15 hr later. The monokine had no effect on the K_m value for 2-deoxy-D-glucose uptake (1.1 mM), while V_{max} was increased from 912 to 2400 pmol/min per mg of protein. The increase was cytochalasin B inhibitable and was dependent on protein synthesis. Photoaffinity labeling and equilibrium binding studies with [^{3}H]cytochalasin B support the hypothesis that this increase in hexose transport was due to an increase in hexose transporters present in the plasma membrane. Purified recombinant interleukin-1α had no effect on hexose transport, whereas purified recombinant cachectin/tumor necrosis factor did stimulate hexose uptake, with half-maximal stimulation occurring at 36 nM. Although cachectin accounts for most of the biological activity associated with the crude monokine preparations, it is not the only monokine capable of inducing glucose transport in L6 cells. Specific immunoabsorption of cachectin/tumor necrosis factor from the crude monokine preparation revealed a monokine that had a similar bioactivity at extremely low concentrations on L6 cells.**

One of the clinical hallmarks of animals with sepsis or endotoxemia is the presence of a catabolic state that can proceed to cachexia, shock, and death (1, 2). Primary among the metabolic sequelae of sepsis are the profound alterations observed in glucose homeostasis (3). This includes an acceleration of glycogenolysis, a depression of glycogen synthesis, biphasic changes in hepatic gluconeogenesis, and enhanced peripheral use of glucose (4–7). Serum glucose concentrations reflect an early hyperglycemia that tends to end in a profound hypoglycemia (3, 6). The massive outpouring of lactic acid, in part, accounts for the fall in blood pH noted in endotoxemia. Secretory proteins of the mononuclear phagocyte system (monokines) have been implicated in the regulation of these events (6, 8–10).

Recently, we studied the effect of monokines on the adipocyte cell line 3T3-L1 (11). These monokines have the ability to suppress the key lipogenic enzymes, lipoprotein lipase, acetyl-CoA carboxylase, and fatty acid synthetase (12). We isolated from this mixture the protein cachectin, which could suppress specifically the mRNAs for a number of adipocyte-specific proteins, rendering the cell unable to synthesize and store triacylglycerol, which led to the induction of a catabolic mobilization state (13, 14). Subsequent studies (15) revealed that cachectin was identical to tumor necrosis factor (TNF) (16). During the past year the biological activities associated with cachectin/TNF have increased significantly (see ref. 17 for a recent review). Of particular interest is the key role that this monokine plays in the metabolic derangement accompanying endotoxic shock. A recent (18) study has shown that injection of recombinant cachectin into rats can evoke many of the physiological and pathological changes noted in experimental animals with endotoxin administration. In addition, interleukin 1α (IL-1α) has been implicated previously in the breakdown of muscle protein (19). Accordingly, it was of interest to determine whether cachectin or IL-1α could induce the changes in muscle metabolism noted in endotoxemia.

We report here that monokines from endotoxin-stimulated RAW 264.7 macrophages stimulate increased glucose uptake and glycogen breakdown in myotubes of the L6 muscle cell line. The increased uptake of glucose was dependent on the synthesis of new glucose transporters and their placement in the cell membrane. Recombinant cachectin/TNF but not recombinant IL-1α exhibited these biological activities. In addition, a new monokine was detected after specific immunoabsorption of cachectin, which had a similar activity on L6 cells.

MATERIALS AND METHODS

Materials. Dulbecco's modified Eagle's medium was purchased from GIBCO. Calf and fetal calf sera were purchased from Sterile Systems (Logan, UT). Nuserum was obtained from Collaborative Research (Waltham, MA). Lipopolysaccharide B (endotoxin) from *Escherichia coli* 0127:B8 was obtained from Difco (Detroit, MI). [^{3}H]-3-*O*-[*methyl*-^{3}H]methyl-D-glucose ([^{3}H]3MeG; 11.6 Ci/mmol; 1 Ci = 37 GBq), 2-deoxy-D-[^{3}H]glucose ([^{3}H]2dG; 30.2 Ci/mmol), [^{3}H]cytochalasin B (15.5 Ci/mmol), and D-[U-^{14}C]glucose 6-phosphate (sodium salt; 125 mCi/mmol) were from New England Nuclear. Recombinant cachectin was prepared and isolated as described (14). Purified recombinant murine IL-1α was the gift of Peter Lomedico (Hoffman–LaRoche, Nutley, NJ). L6 myoblasts, RAW 264.7 macrophages, and L-929 mouse fibroblasts were from the American Type Culture Collection. 2dG, 3MeG, cytochalasin B, enzymes and all other biochemical reagents, unless otherwise specified, were obtained from Sigma.

Culture of L6, RAW 264.7, and L-929 Cells. Murine L6 muscle cells were plated as myoblasts that fused to form multinucleated myotubes exhibiting structural features consistent with skeletal muscle morphology (19, 20). The cells were cultured in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum. RAW 264.7 macrophages were grown and incubated with endotoxin to

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Abbreviations: 2dG, 2-deoxy-D-glucose; 3MeG, 3-*O*-methyl-D-glucose; TNF, tumor necrosis factor; IL-1α, interleukin 1α.

simulate monokine production as described by Mahoney et al. (21). L-929 murine fibroblasts were grown under similar conditions as the L6 cells. Cachectin/TNF cytotoxic activity was measured by using the cytotoxic assay with actinomycin D-treated L-929 cells (22). Cells were incubated at 37°C in humid 5% CO_2/95% air.

Fructose 2,6-bisphosphate levels were determined as described by Van Schaftingen et al. (23). Lactate was measured by the method of Gutmann and Wahlefeld (24). Glycogen was determined in the L6 myotubes by the method of Keppler and Decker (25). Glucose was determined by the glucose oxidase method (26).

Determination of 2dG Uptake. Fully differentiated L6 myotubes were incubated in the presence of increasing amounts of RAW monokines for the indicated times prior to the assay. Uptake of 3MeG and 2dG was measured by a modification of the method of Pekala et al. (27).

To avoid the problem of nonlinearity, which has been ascribed to insufficient ATP levels (28, 29), assays were routinely performed in the presence of 0.1 mM 2dG for a maximum of 5 min. For the kinetic studies, involving higher 2dG concentrations, assay times were shortened to 1 min to reduce nonlinearity to negligible levels. The data obtained in these studies were fitted to the Michaelis–Menten equation by the nonlinear curve-fitting method using the simplex algorithm (30, 31) as suggested by Doud and Riggs (32). Cytochalasin B (10 μM) was added to duplicate monolayers to estimate carrier-independent uptake.

Photoaffinity Labeling. Photoaffinity labeling of L6 cells with [^{3}H]cytochalasin B was performed by a modification of the procedure of Oka and Czech (33). Fully differentiated myotubes were incubated in the presence or absence of conditioned medium from endotoxin-stimulated RAW 264.7 cells (15 μg of protein per ml) for 17 hr. The medium was aspirated off, and the cells were incubated in the presence of [^{3}H]cytochalasin B (15.5 Ci/mmol), unlabeled cytochalasin B at a final concentration of 300 nM, and cytochalasin E at a final concentration of 2 μm. D-Glucose was added to half of the plates at a concentration of 500 mM for determination of nonspecific binding. After an incubation period of 10 min at 37°C, the cells were irradiated at a distance of 26 cm with a 1000-W Porta-cure lamp (American Ultraviolet, Chatham, NJ) through a glass color filter (no. 7-54, Farrand Optical, Valhalla, NY) three times for 10 s with a 10-s rest period in between. After irradiation the medium was aspirated off and rinsed twice with warm Krebs–Ringer solution. Subsequently the cells were scraped in homogenization medium (250 mM sucrose/20 mM Hepes, pH 7.4). The amount of bound radioactivity was determined by liquid scintillation counting.

Preparation of Plasma Membranes and Microsomes. Membrane fractions of L6 myotubes were prepared by using a discontinuous sucrose gradient as described by Grimditch et al. (34). The activity of the plasma membrane marker enzyme γ-glutamyl transpeptidase (35) and the microsomal marker enzyme glucose-6-phosphatase (36) were measured in 1.5-ml fractions. The peak activity of γ-glutamyl transpeptidase was located in the top two fractions, while the peak of glucose-6-phosphatase activity resided in fractions 4 and 5. The plasma membrane fractions 1 and 2 contained <0.85% of the total microsomal marker enzyme activity, while the microsomal membrane fractions 4 and 5 contained ≈3.5% of the total membrane marker activity. The amount of D-glucose-competitive [^{3}H]cytochalasin B binding activity in the isolated fractions was then determined for membrane preparations prepared from control cells and for those that had been exposed to protein (15 μg/ml) from RAW 264.7 monokine preparation for 17 hr. Individual membrane fractions (plasma and microsomal) were diluted 1:3 (vol/vol) with 20 mM Hepes (pH 7.4) and concentrated by sedimentation at 200,000 × g for 24 hr. The pellet was resuspended in 250 μl of cold 50 mM Tris·HCl (pH 7.0). Membrane protein (10–20 μg) was incubated overnight at 4°C in 50 mM Tris·HCl (pH 7.0) containing 76 nM [^{3}H]cytochalasin B and 5 μM cytochalasin E. Identical incubations were carried out in the presence of 500 mM D-glucose for determination of nonspecific binding. After the overnight incubation, membranes were sedimented at 100,000 × g, and bound [^{3}H]cytochalasin B was determined by liquid scintillation techniques.

Immunoabsorption. Cachectin was removed from the crude monokine preparation by using an affinity column of Sepharose-bound polyclonal rabbit antibodies raised against mouse cachectin. Briefly, immunoglobulins from rabbit antiserum were precipitated with 40% saturated $(NH_4)_2SO_4$ (37). The precipitated immunoglobulins were desalted, dissolved in 0.1 M $NaHCO_3$/0.5 M NaCl, pH 8.3, and coupled to cyanogen bromide-activated Sepharose 4B by following the manufacturer's recommended procedure (Pharmacia). A volume of resin equivalent to 3 ml of packed resin was added to an Econo-Column (1 cm × 5 cm, Bio-Rad) and extensively washed with 10 column volumes of phosphate-buffered saline (pH 7.4). Crude monokine preparation (1 ml; 500 μg of protein per ml) was added to the resin and allowed to stand at 5°C. After 30 min the column was centrifuged at 500 × g to recover 100% of the volume added to the column and >98% of the loaded protein. The eluted material was immediately tested for cytotoxic activity on L-929 cells as well as for its ability to stimulate [^{3}H]2dG uptake. The column was subsequently washed again with 10 volumns of phosphate-buffered saline (pH 7.4), and the bound material was eluted with 1 ml of glycine·HCl buffer (pH 2.8) (38). After neutralization with 5 M KOH, the sample was assayed for cytotoxicity on L-929 cells. The pooled material proved to contain at least 80% of the cachectin cytotoxic activity of the original crude monokine preparation.

RESULTS

Monokine Stimulation of 3MeG and 2dG Uptake in L6 Myotubes. When fully differentiated L6 myotubes were exposed to the RAW monokine preparation, a dose-dependent increase in glucose utilization was observed after addition of the monokine preparation. After 48 hr, 15 μg of protein of the crude monokine preparation resulted in an increased glucose extraction from the medium that was 56 ± 3 mg/dl higher than an uptake of 166 ± 3 mg/dl in cells not treated with the monokine. Accompanying this increased utilization was a dose-dependent increase in hexose transport as measured by 2dG transport (Fig. 1). In this experiment the myotubes, which were treated with the RAW monokine preparation 17 hr previously, showed 185% increase in 2dG uptake compared to nontreated cells. Similarly, a dose-dependent increase of 3MeG transport was observed when myotubes were exposed to the RAW monokine 18 hr previously (data not shown). Again a maximum stimulation of 2-fold was observed. Analysis of the myotubes revealed that 80–90% of the intracellular 2dG was phosphorylated, ruling out an underestimation of hexose transport by a depletion of intracellular ATP (39). L6 myotubes treated with the amount of endotoxin found in the crude monokine preparation or in medium from macrophages not stimulated showed 3MeG and 2dG uptake similar to the control cells (data not shown). Moreover, when the crude monokine preparation was examined for the presence of insulin by radioimmunoassay, the results were negative.

The ability of the cells to increase hexose transport in response to the RAW monokines required time and protein synthesis. Fig. 2 records the increase in 2dG uptake over time in L6 myotubes incubated with RAW monokines. Maximum stimulation occurred 12 hr after the addition of the RAW monokines. The addition of the protein synthesis inhibitor

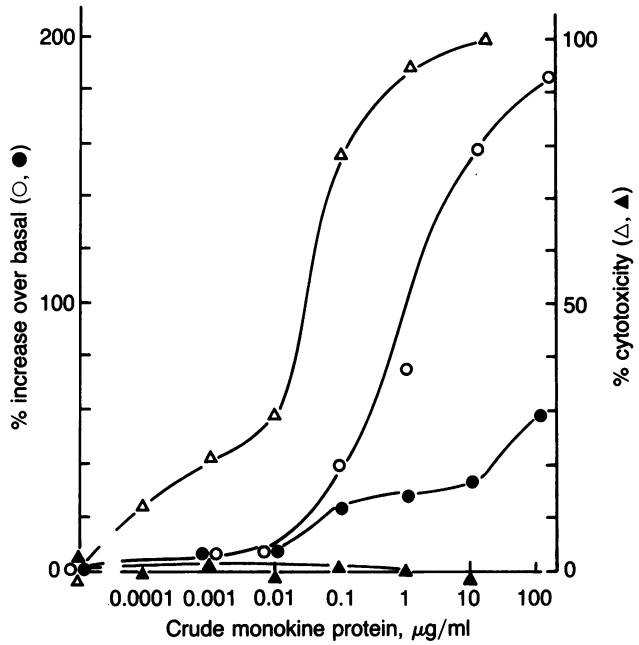

FIG. 1. Dose-dependent increase in [^{3}H]2dG uptake in differentiated L6 myotubes and cytotoxicity on L-929 cells of the crude monokine preparation before and after absorption of cachectin. Induction of [^{3}H]2dG uptake in L6 myotubes after 17 hr of incubation with increasing amounts of crude monokine preparation before (○) and after (●) absorption of cachectin. The results are expressed as the percentage increase over the label incorporated in control cells (2600 cpm/200 μg of protein per well). Cytotoxicity on L-929 cells is shown before (△) and after (▲) absorption of cachectin.

cycloheximide to the L6 cells coincident with the RAW monokine preparation completely blocked the increase in hexose transport without affecting basal 2dG uptake (Fig. 2, see the hatched bar at the 12-hr point).

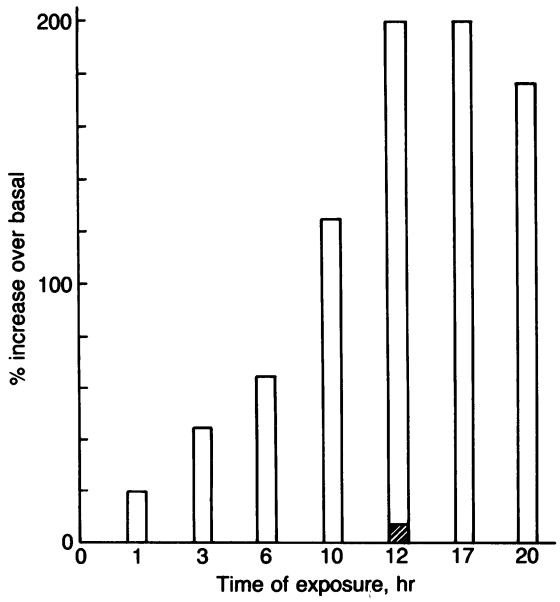

FIG. 2. Time course of the stimulation of [^{3}H]2dG uptake and its dependency on protein synthesis. L6 myotubes were incubated with the crude monokine preparation (15 μg of protein per ml) for the indicated times. Results are expressed as the percentage increase over the control cells (2600 cpm/200 μg of protein per well). Cyclohexamide (11 μM) (▨) blocked the increase in uptake. Cyclohexamide alone had no effect on basal uptake (data not shown). The uptake remained the same in control cells at all the times.

The ability of the RAW monokines to increase the rate of 2dG uptake at different hexose concentrations was assayed. The data were fitted to the Michaelis–Menton equation, and kinetic constants were determined by a nonlinear curve-fitting method using the simplex algorithm (30, 31) as suggested by Doud and Riggs (32). The data indicate that, after exposure to the RAW monokine preparation, no change was observed in the K_m for the system (1.1 mM), while the V_{max} increased from 912 to 2400 pmol/min per mg of protein.

The results above implied that the L6 cells, in response to RAW monokines, produced more glucose transporters. This suggestion was substantiated by determining the amount of [^{3}H]cytochalasin B bound to plasma and microsomal membranes by photoaffinity labeling and equilibrium binding (Table 1). By these two methods, it appears that 17 hr after the exposure of L6 myotubes to RAW monokines there was a 2-fold increase in the number of glucose transporters in the plasma membranes and a 3-fold increase in the microsomal fraction.

Effect of RAW Monokines on Hexose Metabolism in L6 Myotubes. Exposure of the L6 myotubes to the RAW monokine preparation resulted in the rapid depletion of glycogen stores concomitant with an increased production of lactate (Fig. 3). The potent regulator of glycolysis, fructose 2,6-bisphosphate increased rapidly in concentration to nearly 2-fold over basal levels. The time frame for this increase is coincident with that for glycogen depletion and lactate production. The data displayed in Fig. 3 indicate that, at a time when glucose transporter numbers had increased, the glycogen stores began to be replenished.

Effect of Known Monokines on L6 Cells. The addition of recombinant IL-1α, a monokine that shares many biological activities with cachectin (17, 40), did not prompt increased 2dG uptake or lactate production (data not shown). On the other hand, the addition of increasing amounts of recombinant cachectin to the L6 myotubes resulted in a stimulation of 2dG uptake and increased lactate production (Fig. 4). The amount of cachectin necessary to achieve half-maximal stimulation of 2dG transport was 36 nM, a value that is 1000 times more than that necessary to half-maximally suppress lipoprotein lipase in 3T3-L1 cells (11). It should also be noted that, in contrast to the crude monokine preparation, recombinant cachectin can only increase 2dG uptake by 100%.

To determine how much of the biological activity of the crude monokine preparation was due to cachectin, the cachectin was absorbed to specific rabbit anti-mouse cachectin antibodies that were coupled to Sepharose beads. Fig. 1 displays the dose–response curve to the crude monokine preparation before and after absorption of

Table 1. Effect of monokine treatment on the distribution of glucose-competitive cytochalasin B binding between plasma and microsomal membranes

Preparation	Bound [^{3}H]cytochalasin B, pmol/mg of protein	
	Photoaffinity labeling	Equilibrium binding
Control membranes		
Plasma	8.4 ± 1.50	15.4 ± 1.60
Microsomal	3.5 ± 0.30	6.6 ± 0.63
Monokine-treated membranes		
Plasma	18.7 ± 3.79	33.7 ± 4.90
Microsomal	13.6 ± 1.72	20.1 ± 3.50

Fully differentiated L6 myotubes were incubated in the absence or in the presence of the crude monokine preparation (15 μg/ml). After 17 hr of incubation, the monolayers were used either directly for the photoaffinity labeling procedure or for membrane preparation for the equilibrium binding studies as described. The results are means of duplicate experiments ± SEM.

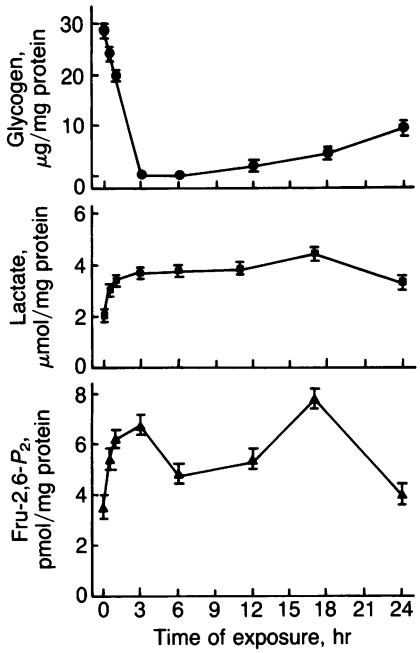

FIG. 3. Effect of the crude monokine preparation on metabolite levels in L6 myotubes. Myotubes were incubated in the presence of 15 μg of protein per ml of crude monokine preparation. At the end of the indicated incubation periods, medium samples were collected to determine lactate. Myotubes were digested with 1 M NaOH to assay fructose 2,6-bisphosphate (Fru-2,6-P_2) levels or scraped in 10% tricholoroacetic acid to measure intracellular glycogen. Results are the mean ± SEM of three experiments performed in duplicate.

cachectin with regard to killing L-929 cells (a cachectin bioassay) and the increase in 2dG uptake in L6 myotubes. It is evident that the antibody removed all of the cachectin bioactivity and most of the response noted with the

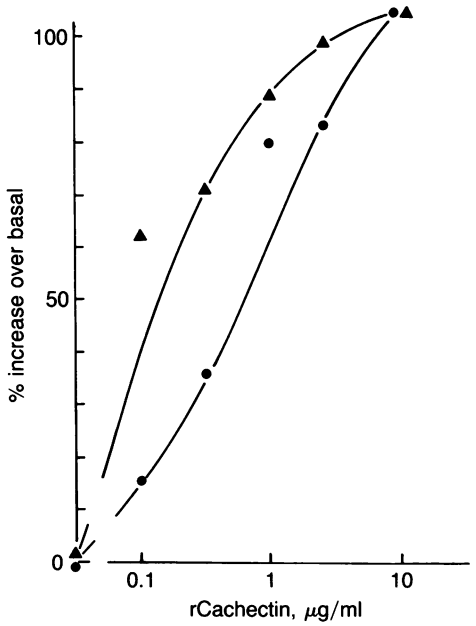

FIG. 4. Dose-dependent increase in [^{3}H]2dG uptake with concomitant increase in lactate production in response to increasing amounts of recombinant human cachectin/TNF (rCachectin). After 19 hr of incubation with different amounts of cachectin/TNF, lactate was assayed in the medium. Identical monolayers were assayed for their ability to take up [^{3}H]2dG as previously described. ●, Glucose uptake; ▲, lactate production. Results are the means of triplicates and are expressed as the percentage increase over results with control cells.

myoblasts. However, an activity persisted in the crude monokine preparation that had a maximum effect of 15–20% at low concentrations of the crude monokine. The nature of this monokine is at present unknown.

DISCUSSION

The present studies show that endotoxin can induce macrophages to secrete monokines capable of modulating hexose utilization by muscle cells. Within minutes after addition of the crude monokine preparation to the myotubes, there is a rapid depletion of intracellular glycogen accompanied by concomitant increases in both lactate and the regulatory metabolite fructose 2,6-bisphosphate. The depletion of intracellular glycogen in L6 myotubes treated with the monokine preparation was followed by an induction of increased uptake of glucose as assessed by the uptake of 3MeG or 2dG, which reached a maximum value at 12 hr. Photoaffinity labeling as well as equilibrium binding studies with [^{3}H]cytochalasin B indicated that this response resulted from an increase in the number of D-glucose-specific transporters in both the plasma and the microsomal fractions. The result is consistent with the observed increase in the V_{max} for 2dG uptake. A possible increase in transporter activity cannot be ruled out; however, the fact that the increase in glucose transporters in monokine-treated cells (Table 1) corresponds to the increase in glucose uptake (Fig. 1) is indirect evidence that monokine-induced transporters account for most of the increase in transport activity. Evidently the increase in the number of glucose transporters is sufficient to provide the cell with appropriate amounts of glucose, since the glycogen stores begin to slowly rise after 12 hr.

Addition of the cloned recombinant monokines IL-1α and cachectin revealed that IL-1α had no activity, whereas cachectin had substantial activity. Whether L6 cells have IL-1α receptors remains to be determined. This may explain the lack of activity of IL-1α on these cells. As little as 100 ng of recombinant cachectin increased the lactate production by ≈60% above control cells and increased 2dG uptake by 15%. However, the maximum increase in either of these parameters was not as high as that observed with the crude monokine preparation. In addition, in contrast to the suppression of lipoprotein lipase in adipocytes, which is inhibited 50% at ≈3 pM, the myotubes require nearly 36 nM to achieve a 50% increase in the uptake of 2dG. These results prompted us to consider the possibility of additional monokines in the crude monokine preparation. Using a rabbit anti-mouse cachectin antibody attached to Sepharose beads, we absorbed the cachectin from the crude monokine preparation (Fig. 1). This procedure completely removed all of the cachectin as evidenced by the loss of the killing activity of L-929 cells. However, a small amount of activity (about 15% of the total) remained in the crude monokine preparation. The nature of this protein, which is maximally active at low concentrations, is not known but presumably reflects a monokine that has overlapping activity with cachectin. Since cachectin cannot achieve the same degree of induced 2dG uptake as the crude monokine preparation, it also implies that an additional monokine is synergistic with cachectin in eliciting this response.

The mechanism by which monokines promote the synthesis of glucose transporters in L6 myotubes is not known. It contrasts, for example, with the current theory of insulin action in which preexisting glucose transporters are rapidly mobilized from an intracellular pool. One possible explanation is that the monokine activates a mechanism that dissipates energy in the cell, which leads to the loss of energy stores and the demand for more glucose. Cachectin, for example, has been shown recently to be responsible for the depolarization of muscle cells *in vivo* and *in vitro* (41). It is not

known at present whether this depolarization reflects an increase in cell permeability or a decrease in pumping mechanisms. Therefore, the energy consumed in the cachectin-treated muscle cell may reflect the attempt of the cell to return the membrane potential to normal. This hypothesis is worthy of further investigation because it may give insight into the effects of cachectin on other cells as well as explain its ability to kill certain transformed cells.

The increased glucose utilization observed with the myotubes *in vitro* in response to macrophage monokines presumably is analogous to the increased peripheral glucose utilization that is associated with late-phase hypoglycemia of sepsis or endotoxemia. Within 3 hr after endotoxin administration, there is a total depletion of glycogen and increased lactate output (42). As evidenced by the current work, the interplay of cachectin and other monokines may be responsible for this physiologic response to endotoxin and sepsis. The possibility that cachectin and these other monokines prompt the wasting of muscle mass associated with cachexia by increasing energy utilization also needs further study.

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