Induction of Muscle Protein Degradation and Weight Loss by a Tumor Product

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ABSTRACT

Splenocytes from mice bearing a cachexia-inducing tumor (MAC16) have been fused with mouse myeloma cells to produce hybrids, which have been cloned to produce antibody reactive to a material which copurified with a lipid-mobilizing factor isolated from the same tumor. The monoclonal antibody has been used to investigate factors potentially involved in the development of cachexia. The major protein detectable by immunoprecipitation of a partially purified lipid-mobilizing factor was Mr 69,000, whereas Western blotting showed two bands of Mr 69,000 and Mr 24,000. Although the monoclonal antibody did not neutralize lipid-mobilizing activity in an in vitro assay, it did neutralize a serum factor capable of protein degradation in isolated gastrocnemius muscle. Affinity purification of MAC16 tumor homogenates using the monoclonal antibody yielded two immunoreactive bands of Mr 69,000 and Mr 24,000, which were further fractionated on a hydrophobic column (C8). This material was capable of inducing tyrosine release from isolated gastrocnemius muscle, and the effect could be blocked with the monoclonal antibody. The two immunoreactive bands from the hydrophobic column were capable of inducing weight loss in mice, whereas nonimmunoreactive fractions had no effect on body weight. The Mr 24,000 species had a unique amino acid sequence, whereas the Mr 69,000 species gave the same sequence as the Mr 24,000 material, together with that for albumin. The Mr 24,000 species contained carbohydrate, and lectin blotting showed a strong reaction with wheat germ and Erythrina cristagalli agglutinins. This suggests that the material is a glycoprotein or proteoglycan that shows strong binding affinity for albumin, possibly through the carbohydrate residues.

INTRODUCTION

Cancer cachexia is a complex metabolic problem characterized by weight loss with depletion of both skeletal muscle and adipose tissue mass (1). Although anorexia commonly accompanies cachexia, weight loss can occur even in patients with a normal energy intake (2). In addition, many patients either maintain body weight or lose weight while receiving calories that would be predicted to result in weight gain (3). These effects arise from an increased resting energy expend-iture in some cancer patients (4). The absence of a direct nutritional cause has led to numerous studies into the identification of mediators of the cachectic process.

Such mediators might be expected to have diverse biological effects in view of the multifunctional host impairment observed in cachexia. A number of studies have suggested cytokines, such as tumor necrosis factor-α and IL-1 and IL-6, as mediators of the process of cachexia. These molecules do not initiate direct lipid catabolism from adipose tissue but rather indirectly by inhibition of the enzyme lipoprotein lipase, preventing adipocytes from extracting fatty acids from plasma lipoproteins and resulting in a net flux of lipid into the circulation (5). Although capable of inducing protein degradation in vitro (6, 7), in vitro incubation of skeletal muscle with the cytokines failed to show a direct catabolic effect (7, 8), suggesting that the in vivo effects may be triggered by an unknown intermediary factor. This view was substantiated with a study of cancer patients with weight loss that showed in vitro proteolysis-inducing factors in the serum not found in healthy subjects (9). The bioactivity in 20% of the samples was partially abrogated by antibodies to recombinant IL-1, suggesting that the accelerated breakdown of protein may be mediated by IL-1 in cooperation with other unidentified factors.

Our own studies using the MAC16 murine cachexia model have also provided evidence for a proteolysis-inducing factor in the serum of animals with weight loss (10). Protein degradation was specific to the cachetic state since similar material was not present in the serum of mice bearing the MAC13 tumor, which does not produce weight loss (11). Recently, we described a polyclonal antibody present in the serum of mice bearing the MAC16 tumor but absent from mice bearing the MAC13 tumor, which showed immunoreactivity towards a material of apparent molecular weight of Mr 24,000, which copurified with a lipid-mobilizing factor associated with cachexia (12). This report describes the production of a monoclonal antibody to this material using splenocytes from mice bearing the MAC16 tumor and the evaluation of this antibody in the purification of a potential cachectic factor.

MATERIALS AND METHODS

Chemicals. RPMI 1640 tissue culture media and myocyte plus FCS were purchased from GIBCO-BRL (Scotland, United Kingdom). Hypoxanthine-aminopterin-thymidin media supplement and BM-Condimed HI were purchased from Boehringer Mannheim UK (Lewes, East Sussex, United Kingdom), and the protein A-Sepharose was purchased from Sigma Chemical Co. Ltd. (Poole, Dorset, United Kingdom).

Preparation of Splenocytes for Fusion. Animals were transplanted with the MAC16 tumor and assessed for the development of antibodies to the lipid-mobilizing factor (12) using serum isolated from blood removed from the tail vein. Suitable animals were killed, and the spleens were removed under aseptic conditions and placed in a 100-mm Petri dish containing 10 ml of RPMI 1640 without serum and prewarmed to 37°C. The spleens were teased apart until most of the cells had been released. The cells were then transferred into a sterile centrifuge tube, leaving behind the larger pieces of tissue. The residue was washed with an additional 10 ml of RPMI 1640, and the washings were combined. The cell suspension was allowed to stand for 2 min, after which the supernatant was removed from the sediment and transferred to a fresh centrifuge tube. These cells were then ready for fusion.

Preparation of Myeloma Cells for Fusion. Mouse BALB/c myeloma P3 × 63Ag 8.653 was obtained from the European Collection of Animal Cell Cultures (Porton Down, Wiltshire, United Kingdom). Cells were passaged in RPMI 1640 supplemented with 10% myocyte plus FCS. One day prior to the fusion, cells were diluted to a concentration of 5 × 10⁶/ml, and on the day of fusion, cells were diluted with an equal volume of medium supplemented with 20% myocyte plus FCS.

Fusion. The splenocytes and the myeloma cells were washed in separate centrifuge tubes with RPMI 1640 without serum. After the wash, 10⁶ mouse splenocytes were mixed with 2 × 10⁶ myeloma cells and centrifuged together at 400 × g for 5 min. The supernatant was completely removed, and 1.5 ml of polyethylene glycol 1500 (w/v) prewarmed to 37°C was added to the pellet drop by drop over a period of 1 min while the cells were continually stirred. This was followed by the addition of 1 ml of RPMI 1640 prewarmed to 37°C over the next minute, followed by an additional 9 ml of RPMI 1640 over the

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1 This work was supported by Cancer Research Campaign Grant SP1518.
2 To whom requests for reprints should be addressed.
3 The abbreviation used is: IL,—interleukin.
at 37°C under an atmosphere of 5% CO₂ in air. Cells were routinely subcultured every 4 days. For maximal production of monoclonal antibody, cells were left for 7 days, and a cell-free supernatant was obtained by centrifugation at 3000 rpm for 5 min using a bench centrifuge. The supernatant was adjusted to pH 8 with 1M Tris, and the antibody solution was slowly passed through a protein A-Sepharose column (binding capacity ~35 mg human IgG/ml). The protein-A column was washed with 60-column volumes of 10 mM Tris (pH 8.0), followed by 30-column volumes of 10 mM Tris (pH 8.8). The antibody was eluted slowly with 100 mM glycine (pH 3), and fractions of 0.5 ml were collected. The fractions were neutralized with 50 µl of 1M Tris (pH 8). The protein concentration of the individual fractions was determined by the Bio-Rad method, and fractions containing protein were pooled. The protein was concentrated by a diaflo ultrafiltration membrane (YM10) in a stirred ultrafiltration cell (Amicon Inc., Beverly, MA). The concentration of monoclonal antibody was found to be 8.6 mg per 1 ml of tissue culture supernatant. A control mouse IgG2a raised against a rat immunoglobulin was kindly supplied by M. Valeri (Institute of Cancer Research, Surrey, United Kingdom).

Propagation of Hybridomas and Isolation of Monoclonal Antibody. The hybridomas were routinely passaged in RPMI 1640 containing 25 mM HEPES, 5% l-glutamine, and 10% fetal bovine serum (Myocyte Plus) under an atmosphere of 5% CO₂ in air. Cells were routinely subcultured every 4 days. For maximal production of monoclonal antibody, cells were left for 7 days, and a cell-free supernatant was obtained by centrifugation at 3000 rpm for 5 min using a bench centrifuge. The supernatant was adjusted to pH 8 with 1M Tris, and the antibody solution was slowly passed through a protein A-Sepharose column (binding capacity ~35 mg human IgG/ml). The protein-A column was washed with 60-column volumes of 10 mM Tris (pH 8.0), followed by 30-column volumes of 10 mM Tris (pH 8.8). The antibody was eluted slowly with 100 mM glycine (pH 3), and fractions of 0.5 ml were collected. The fractions were neutralized with 50 µl of 1M Tris (pH 8). The protein concentration of the individual fractions was determined by the Bio-Rad method, and fractions containing protein were pooled. The protein was concentrated by a diaflo ultrafiltration membrane (YM10) in a stirred ultrafiltration cell (Amicon Inc., Beverly, MA). The concentration of monoclonal antibody was found to be 8.6 mg per 1 ml of tissue culture supernatant. A control mouse IgG2a raised against a rat immunoglobulin was kindly supplied by M. Valeri (Institute of Cancer Research, Surrey, United Kingdom).

Purification of Lipid-mobilizing Factor from the MAC16 Tumor. This was carried out as described previously (12).

The hybridomas were routinely passaged in RPMI 1640 containing 25 mM HEPES, 5% l-glutamine, and 10% fetal bovine serum (Myocyte Plus) under an atmosphere of 5% CO₂ in air. Cells were routinely subcultured every 4 days. For maximal production of monoclonal antibody, cells were left for 7 days, and a cell-free supernatant was obtained by centrifugation at 3000 rpm for 5 min using a bench centrifuge. The supernatant was adjusted to pH 8 with 1M Tris, and the antibody solution was slowly passed through a protein A-Sepharose column (binding capacity ~35 mg human IgG/ml). The protein-A column was washed with 60-column volumes of 10 mM Tris (pH 8.0), followed by 30-column volumes of 10 mM Tris (pH 8.8). The antibody was eluted slowly with 100 mM glycine (pH 3), and fractions of 0.5 ml were collected. The fractions were neutralized with 50 µl of 1M Tris (pH 8). The protein concentration of the individual fractions was determined by the Bio-Rad method, and fractions containing protein were pooled. The protein was concentrated by a diaflo ultrafiltration membrane (YM10) in a stirred ultrafiltration cell (Amicon Inc., Beverly, MA). The concentration of monoclonal antibody was found to be 8.6 mg per 1 ml of tissue culture supernatant. A control mouse IgG2a raised against a rat immunoglobulin was kindly supplied by M. Valeri (Institute of Cancer Research, Surrey, United Kingdom).

Fig. 1. Immunoprecipitation of lipid-mobilizing factor with mouse monoclonal antibody. The details of the procedure are given in the legend to Table 1. A, Coomassie blue staining of a 15% SDS-PAGE gel of immunoprecipitated material (Lanes 2 and 3) compared with the monoclonal antibody (Lanes 4 and 5). Lane 1 contains the molecular markers. B, immunoblot analysis of immunoprecipitated material using the MAC16 monoclonal antibody (Lane 2). Lane 1 contains the molecular markers. C, Western blot of ELISA-positive fractions eluted from the affinity column (Table 3). Lane 1, molecular markers; Lanes 2–4, ELISA-positive fraction. D, silver staining of a 15% SDS-PAGE gel of immunoreactive fraction isolated by affinity chromatography of a MAC16 tumor homogenate (Table 3).
at 214 nm, and protein peaks were collected into microfuge tubes containing 0.1 ml of 0.1 M carbonate/bicarbonate buffer (pH 9.5). The acetoneint was removed under a stream of nitrogen, and the volume was reduced to 0.1 ml. The immunoreactivity of the individual protein peaks was determined by an ELISA plate assay.

**ELISA Plate Assay.** Samples were divided into two and immobilized on a 96-well polystyrene plate assay (Costar, Cambridge, MA) overnight at 4°C. The liquid was removed by aspiration, and the wells were washed three times with PBS + 0.1% Tween-20 (200 μl). Blocking solution (200 μl of PBS containing 0.1% Tween 20 and 3% BSA) was added to the wells, and the plate was incubated for 2 h at 37°C. One-half of the sample was incubated with the monoclonal antibody (10 μg/ml) in blocking solution (100 μl) for 1 h at room temperature, while the other half was incubated in the same solution, but in the absence of the antibody. After removal of the antibody solution, the wells were washed six times before the addition of a protein A peroxidase conjugate (Sigma Chemical Co., Poole, United Kingdom). The plates were incubated for 30 min. The reaction was terminated by the addition of 0.2 M H₂SO₄ (50 μl/well), and the absorbance was determined at 492 nm using a microplate reader (Anthos Labtec Instruments).

**Western Blotting.** For immunoblotting, gels were transferred to nitrocellulose membranes ( Hoefer Scientific Instruments, San Francisco, CA) that had been blocked with 5% Marvel in 0.1% Tween 20 in PBS at 4°C overnight. The membranes were washed for 15 min and five for 5 min in Tween/PBS and further incubated in the Tween/PBS containing 1.5% Marvel and 10 μg/ml of the monoclonal antibody for 1 h at room temperature. After being washed three times as above, the filters were incubated for 1 h with protein A peroxidase conjugate at a 1:5000-fold dilution, followed by one 15-min wash and four 5-min washes with 0.5% Tween 20 in PBS. Bands were detected with an emission chemiluminescence (ECL) system (Amersham, Buckinghamshire, United Kingdom). The Western Blot was visualized with the aid of a standard color-detection scheme for horseradish peroxidase, which makes use of 4-chloro-1-naphthol and hydrogen peroxide.

**Measurement of Protein Degradation.** Female NMRI mice were killed by cervical dislocation, and their gastrocnemius muscles were quickly ligated, dissected out, and placed in ice-cold isotonic saline. To minimize diurnal variation and to assure that animals were in the fed state, all animals were sacrificed between 9:00 and 10:00 am. The muscles were blotted, weighed, and carefully tied via tendon ligatures to stainless steel incubation supports (13). This prevents contraction and improves protein balance and energy status (14). Protein degradation was measured by tyrosine release, since tyrosine rapidly equilibrates between intracellular pools and the medium and is neither synthesized nor degraded. Muscles were preincubated in RPMI 1640 (3 ml) lacking phenol red in the presence of serum (280 μl) for 30 min at 37°C in an atmosphere saturated with O₂:CO₂ (19:1). The muscles were rinsed and incubated for an additional 2 h in Krebs-Hensel bicitrate buffer containing 6 mM β-glucose, 1.2 mg/ml BSA and 130 μg/ml cycloheximide with continuous gassing. At the end of the incubation, the buffer was removed, deproteinized with ice-cold 3% trichloroacetic acid (0.2 ml), and centrifuged at 2800 × g for 10 min; then the supernatant was used for the measurement of tyrosine by a fluorimetric method (15) at 570 nm on a Perkin-Elmer LS-5 luminescence spectrometer.

**Gel Electrophoresis.** Gels were prepared according to the method of Laemmli (16) and consisted of a 5% stacking gel and a 15% resolving gel. Molecular weight standards used were rabbit muscle myosin (M, 205,000), phosphorylase b (M, 97,000), BSA (M, 66,000), ovalbumin (M, 45,000), carbonic anhydrase (M, 29,000), trypsin inhibitor (M, 20,000), and α-lactalbumin (M, 14,200) and were purchased from Sigma Chemical Co. Ltd.

**Determination of Immunoglobulin and Subclass.** The class and subclass of the monoclonal antibody was determined using a mouse hybridoma subtyping kit (Boehringer Mannheim) according to the manufacturer’s instructions.

**Body Composition Analysis.** For the determination of the total carcass fat and water content, each carcass was placed in an oven at 80°C until constant weight was reached. Carcasses were then reweighed, and the total fat content was determined by the method of Lundholm et al. (17). Lipids were extracted from the whole carcass with chloroform:methanol (1:1), ethanol-acetone (1:1), and then pure ether, which was allowed to evaporate. Water content was calculated from the wet and dry weights.

**Statistical Analysis.** Results are expressed as means ± SEM. Comparison of multiple groups has been made by ANOVA.

**RESULTS**

Using subclass-specific immunoglobulin conjugates from goat, the monoclonal antibody isolated from mice bearing the MAC16 tumor was determined to be of the IgG2a subclass, and a monoclonal antibody of the same subclass was used as a control. To determine if there was any interaction between the monoclonal antibody and lipid-mobilizing activity, material was purified from the solid MAC16 tumor as described (12), mixed with monoclonal antibody bound to protein A-Sepharose, and incubated with agitation overnight at 4°C. After pelleting by centrifugation, no lipid-mobilizing activity could be detected in the supernatant fluid (Table 1). In contrast, incubation with a control monoclonal antibody had no effect on lipid-mobilizing activity. Analysis of the precipitated protein using SDS-PAGE and staining with Coomassie blue gave evidence for one additional band.

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**Table 2** Effect of serum on tyrosine release from isolated gastrocnemius muscle

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Tyrosine (nmol/mg/2 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mouse serum</td>
<td>39 ± 2</td>
</tr>
<tr>
<td>Serum from cachetic MAC16 mice</td>
<td>54 ± 6*</td>
</tr>
<tr>
<td>Serum from cachetic mice + MAC16 monoclonal antibody</td>
<td>41 ± 3*</td>
</tr>
<tr>
<td>Cachetic mouse serum + non-specific IgG2a</td>
<td>47 ± 2</td>
</tr>
<tr>
<td>Normal mouse serum + antigen†</td>
<td>56 ± 5*</td>
</tr>
<tr>
<td>Normal mouse serum + antigen + MAC16 monoclonal antibody</td>
<td>21 ± 2*</td>
</tr>
<tr>
<td>Normal mouse serum + antigen + non-specific IgG2a</td>
<td>54 ± 3</td>
</tr>
</tbody>
</table>

* P < 0.05 when compared with normal mouse serum.
* P < 0.01 when compared with serum from cachetic mice alone.
† Antigenic material was purified from the MAC16 tumor as described in Table 3.
* P < 0.01 when compared with normal mouse serum + antigen.

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**Table 3** Purification of cachexia-inducing material from the MAC16 tumor

<table>
<thead>
<tr>
<th>Stage</th>
<th>Protein (mg)</th>
<th>Protein recovery (%)</th>
<th>A₄₀₀ units</th>
<th>Recovery (%)</th>
<th>Specific activity units/mg</th>
<th>Purification-fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tumor homogenate</td>
<td>3160</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>40% (NH₄)₂SO₄</td>
<td>113</td>
<td>5.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Affinity</td>
<td>0.256</td>
<td>0.012</td>
<td>43</td>
<td>168</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C₈ tlp 55% CH₃CN</td>
<td>1.2 × 10⁻⁴</td>
<td>3.8 × 10⁻⁶</td>
<td>8.6</td>
<td>29</td>
<td>71667</td>
<td>427</td>
</tr>
<tr>
<td>81% CH₃CN</td>
<td>0.124</td>
<td>4 × 10⁻³</td>
<td>3.8</td>
<td>9</td>
<td>31</td>
<td>0.18</td>
</tr>
</tbody>
</table>

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of $M_r$ 69,000 (Fig. 1A) in addition to the antibody heavy and light chains. Western blotting of immunoprecipitated material showed two bands of $M_r$ 69,000 and 24,000 (Fig. 1B). Thus, although the antibody was cloned to material of $M_r$ 24,000 (12), it was also capable of detection of higher molecular weight material (Table 1). Preincubation of MAC16 tumor-derived lipid-mobilizing factor with monoclonal antibody at 4°C overnight failed to prevent the induction of lipolysis in isolated murine adipocytes, suggesting that it is not a neutralizing antibody for this activity.

Since cachexia involves depletion of both adipose tissue and skeletal muscle mass, the effect of the MAC16 monoclonal antibody on tyrosine release from isolated gastrocnemius muscle was also investigated. As reported previously (11), serum from mice bearing the MAC16 tumor caused an increased protein degradation compared with serum from nontumor-bearing animals (Table 2), and this effect was completely abolished by prior incubation of the serum with the MAC16 monoclonal antibody while being unaffected by a nonspecific IgG2a. This suggests that this antibody is directed toward material capable of inducing muscle proteolysis and that this material must be closely associated with material capable of causing lipid mobilization.

To determine the nature of the immunoreactive material, a purification scheme was devised that involved an initial ammonium sulfate fractionation of the MAC16 tumor homogenate, followed by an affinity purification with monoclonal antibody coupled to Affi-gel HZ. This matrix couples the antibody through aldehyde groups formed by oxidation of the carbohydrate residues to hydrazide groups on the gel, allowing the correct orientation of the antibody and resulting in increased binding capacity per coupled IgG. Bound material was eluted from the column with glycine HCl (pH 2.5), and fractions reactive with the antibody were determined by an ELISA plate assay (Table 3). Western blotting of such fractions showed two immunoreactive bands of $M_r$ 69,000 and $M_r$ 24,000 (Fig. 1C), which were the main protein bands detected by silver stain after affinity chromatography (Fig. 1D). Bioactivity of the antigenic material was determined by tyrosine release from isolated gastrocnemius muscle (Table 2). This showed that when antigenic material was added to the serum from nontumor-bearing animals, protein degradation was increased to the level observed with serum from MAC16 tumor-bearing mice with cachexia. The elevation in tyrosine release could be inhibited by preincubation with the MAC16 monoclonal antibody prior to the addition to the isolated gastrocnemius muscle, while being unaffected by a nonspecific IgG2a monoclonal antibody.

Further fractionation of affinity-purified material was achieved using reverse phase hydrophobic chromatography with a C4 column and a gradient from 5 to 100% acetonitrile in water containing 0.06% trifluoroacetic acid (Fig. 2A). Immunologically reactive material eluted

### Table 4 Effect of purified antigen on body weight of female NMRI mice, food and water intake, and plasma metabolic levels

<table>
<thead>
<tr>
<th>Acetonitrile (%)</th>
<th>Weight loss (g)</th>
<th>Food intake g/mouse</th>
<th>Water (ml/mouse)</th>
<th>Glucose mM</th>
<th>Triglyceride mM</th>
<th>Fatty acid mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>56</td>
<td>2.03 ± 0.21</td>
<td>2.73</td>
<td>1.3</td>
<td>5.9 ± 0.2</td>
<td>0.80 ± 0.1</td>
<td>0.97 ± 0.08</td>
</tr>
<tr>
<td>58</td>
<td>0.96 ± 0.29</td>
<td>2.98</td>
<td>1.5</td>
<td>6.4 ± 0.3</td>
<td>0.79 ± 0.19</td>
<td>0.72 ± 0.02</td>
</tr>
<tr>
<td>78</td>
<td>1.84 ± 0.38</td>
<td>3.23</td>
<td>1.4</td>
<td>6.3 ± 0.2</td>
<td>0.49 ± 0.08</td>
<td>1.16 ± 0.12</td>
</tr>
<tr>
<td>83</td>
<td>0.80 ± 0.28</td>
<td>2.33</td>
<td>1.2</td>
<td>6.0 ± 0.4</td>
<td>0.59 ± 0.13</td>
<td>0.90 ± 0.13</td>
</tr>
<tr>
<td>86</td>
<td>0.80 ± 0.17</td>
<td>2.84</td>
<td>1.7</td>
<td>7.5 ± 0.7</td>
<td>0.97 ± 0.11</td>
<td>0.66 ± 0.08</td>
</tr>
<tr>
<td>88</td>
<td>0.39 ± 0.22</td>
<td>2.69</td>
<td>2.2</td>
<td>6.0 ± 0.3</td>
<td>0.77 ± 0.18</td>
<td>0.63 ± 0.05</td>
</tr>
</tbody>
</table>

* Decrease in body weight of female NMRI mice after administration of fractions eluted from the C4 column. To each fraction was added PBS (3 ml), and the samples were placed under a steady stream of nitrogen for 40 min to remove the acetonitrile. The fractions were concentrated by ultrafiltration through an Amicon filtration cell containing a membrane filter with a molecular weight cutoff of 10,000 against PBS without calcium and magnesium, followed by two washes with the same buffer. Portions (150 μl, 5 μg protein) were injected into the tail vein of five female NMRI mice at 1.5-h intervals (10.30, 12.00, 13.30, and 15.00 h). Animals were weighed before each injection, with the final determination at 24 h. Results are for mice 24 h after the first injection and are the mean ± SEM for four to six animals per group.

* $P = 0.001$ from 86 and 88% acetonitrile by one-way ANOVA.

* $P = 0.03$ from 86 and 88% acetonitrile by one-way ANOVA.

* $P = 0.01$ from 86 and 88% acetonitrile by one-way ANOVA.
Fig. 3. Amino acid sequence of p24 antigen.

This confirms that the antigen is capable of producing cachexia. There was no significant change in food and water consumption between the individual groups (Table 4), but there was an elevation of plasma free fatty acids in the groups administered material eluting at 55 and 80% acetonitrile.

Amino acid sequence analysis of the M, 24,000 material eluting from the Cs column with 55% acetonitrile showed a single species of 18(20) amino acids (Fig. 3). No further sequence information could be obtained. Material eluting at 80% acetonitrile gave the same amino acid sequence, together with the sequence for albumin. This suggests that the immuno-reactive band of M, 69,000 is albumin bound to the M, 24,000 material. Both bands stained for carbohydrate using the digoxigenin glycan detection kit (Fig. 4A). Lectin blotting studies on the pure M, 24,000 component showed a strong reaction with Triticum vulgaris wheat germ agglutinin, which has specificity predominantly for N-acetylgalcosamine (Fig. 4B). A strong reaction was also obtained with Erythrina carpogii agglutinin, which has specificity for Gal β(1→4)N-acetylgalcosamine. Neither Con A, which has specificity for mannose, or Tetragonolobus purpureas agglutinin, which has specificity for fucose, gave a reaction. These results suggest that the material of M, 24,000 is a glycoprotein or proteoglycan, which shows strong binding affinity to albumin, possibly through the carbohydrate residues.

DISCUSSION

Previous studies with the cachexia-inducing murine MAC16 tumor model have provided evidence for circulatory tumor products capable of direct catabolism of both skeletal muscle and adipose tissue using in vitro bioassays (18). Such catabolic factors were not present in the circulation of a related tumor, MAC13, which does not induce cachexia. Recently, we reported (12) that mice transplanted with the MAC16 tumor and with delayed weight loss contained in their serum antibodies that interacted with a material of M, 24,000, which copurified with a lipid-mobilizing factor. Such antibodies were not in the serum of mice transplanted with the MAC13 tumor, suggesting that the antibodies were directed to the induction of cachexia rather than the tumor itself. The present study describes the cloning of such antibodies using splenocytes from mice transplanted with the MAC16 tumor and the use of such antibodies to characterize factors potentially responsible for the cachexia.

The major protein produced by immunoprecipitation of a purified lipid mobilizing factor was of M, 69,000, while Western blotting indicated immunoreactive bands at M, 69,000 and M, 24,000. Masuno et al. (19, 20) also reported the isolation of a lipolytic factor (toxohormone-L) from the ascites fluid of patients with hepatoma (19) and mice with sarcoma 180 (20) of M, 70,000. Although not further characterized in terms of structure, this material was similar to the lipid-mobilizing factor produced by the MAC16 tumor in that both were acidic (pI < 4) and eluted from a DEAE-cellulose column between 0.14 and 0.18 M salt (12).

Although the monoclonal antibody was incapable of neutralizing lipid-mobilizing activity in an in vitro assay, it was capable of complete inhibition of the enhanced protein degradation in isolated gastrocnemius muscle induced by serum from mice bearing the MAC16 tumor, thus providing some evidence for a relationship between these two activities. Affinity purification of homogenates of the MAC16...
tumor yielded immunologically reactive material of $M_r$ 69,000 and $M_r$ 24,000, which could be further fractionated using hydrophobic chromatography. This material was capable of stimulating protein degradation in the gastrocnemius muscle assay in a specific manner that could be inhibited by the MAC16 monoclonal antibody.

Amino acid sequence analysis confirmed that the material of $M_r$ 24,000 had a unique sequence and showed no homology with any of the recognized cytokines. Material of $M_r$ 69,000 contained albumin and the same sequence as $M_r$ 24,000. This suggests that binding to albumin is very strong, since complete dissociation was not achieved, even after fractionation using acetonitrile and trifluoroacetic acid. Attempts were made to obtain further sequence information by proteolytic digestion of the material of $M_r$ 24,000. The predominant sequence after tryptic digestion was as shown in Table 5, with no evidence for another species. A low level of non-peptide material was observed. A similar result was obtained after overnight digestion with chymotrypsin, which again only gave evidence for the NH$_2$-terminal sequence. Digestion with V8 protease gave five prominent peaks, three of which corresponded to the NH$_2$-terminal sequence and two to V8 autodigestion products. This suggests that the material of $M_r$ 24,000 is resistant to proteolytic digestion or that no further structural information is present. This abnormal behavior may be explainable by the presence of carbohydrate. Although the material is resistant to digestion by proteolytic enzymes, degradation is achievable using chondroitinase ABC and AC, suggesting that it may be a sulfated heteropolysaccharide. A recent study indicated that cell bound albumin bound strongly to peptidoglycan and sulfated heparinoids to form a complex of Mr 70,000, which migrated as a single band on PAGE (21).

The role of this material in cancer cachexia is supported by an in vivo study demonstrating that immunoreactive material eluting from the hydrophobic column was capable of inducing weight loss. This effect occurred without a reduction in food and water intake, as is found in animals bearing the MAC16 tumor (18). In this murine model, the energy expenditure has been shown to increase with increasing weight loss and since food intake remains constant, a negative energy balance results (22). Thus, the material of $M_r$ 24,000 possibly produces weight loss by increasing the energy expenditure. However, the mechanism by which this material produces cachexia remains to be determined.

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Induction of Muscle Protein Degradation and Weight Loss by a Tumor Product

Penio T. Todorov, Trudi M. McDevitt, Peter Cariuk, et al.

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