Folding of iso-2 at high pH proceeds by passing through a state that takes on (nativelike) structure absent in alkaline iso-2. Thus, our results violate one of the principal tenets of the puzzle model: that structure found in intermediates be a subset of structure in the thermodynamically favored product of folding. Our results do not prove that the entire folding process follows a sequential pathway but do show that the final stages of folding to either native or alkaline iso-2 are dominated by native or nativelike species.

Conclusions

Folding to the alkaline form of iso-2 cytochrome *c* occurs largely through native or nativelike species under conditions where the nonnative alkaline structure is the thermodynamically favored product. This shows that nativelike species play a major role in directing the final stages of folding to a nonnative state.

Acknowledgments

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Identification of a Novel Serum Protein Secreted by Lung Carcinoma Cells

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ABSTRACT: The murine anti-human lung tumor monoclonal antibody L3 recognizes antigens found both in the medium of cultured carcinoma cells and in normal human serum. Sequential immunoprecipitation experiments indicate that the L3 antigen is also recognized by a previously described monoclonal antibody directed against a melanoma-associated antigen [Natali, P. G., Wilson, B. S., Imai, K., Bigotti, A., & Ferrone, S. (1982) *Cancer Res. 42*, 583–589]. This antibody precipitated a M_r 76 000 glycoprotein from metabolically labeled extracts of the lung carcinoma cell line Calu-1 and a M_r 94 000 glycoprotein from labeled culture medium. Pulse–chase experiments suggested a precursor–product relationship between these molecules. Analysis of glycosidase sensitivities of the two forms indicated that maturation of carbohydrate side chains correlated with the apparent increase in molecular weights. L3 antigenic activity, measured in a competitive radiometric cell binding assay, was purified more than 90-fold from serum-free medium of Calu-1 cells and more than 3000-fold from normal human serum. The major immunoreactive components purified from culture medium and serum were identical with respect to apparent molecular weight, electrophoretic mobility, p*I*, glycosidase sensitivity, and V8 protease fingerprints. In addition, the sequence of the amino-terminal 16 N-terminal amino acid residues of the major immunoreactive species from both sources was identical. The properties of the L3 antigen did not correspond to those of any known protein, suggesting that this serum protein has not been previously characterized.

The immunological detection of tumor-associated antigens in patient serum can provide clinically useful data for monitoring malignant disease. α-Fetoprotein, an embryonic analogue of serum albumin, is diagnostic for hepatic tumors when found at elevated levels in adult serum, and serum levels of carcinoembryonic antigen (CEA) are widely used to monitor

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cancers of the colon and other organs [reviewed by Chu (1982)]. More recently developed monoclonal antibody based diagnostic tests have also been reported for cancers of colon (Koprowski et al., 1982), ovary (Bast et al., 1983), breast (Hayes et al., 1985; Paspidero et al., 1984; Burchell et al., 1984), and pancreas (Metzgar et al., 1984).

Monoclonal antibodies with specificity for antigens associated with a number of tumor types have been described, but the proportion of these that recognize molecules released in significant amounts into the blood is small. This most likely results from the fact that many of the tumor-associated antigens that have been described are molecules firmly associated with the cell membrane. Although some membrane antigens may be shed by tumor cells, it would seem likely that the research for new serum markers should be focused specifically on antigens whose biological fate is to be released by tumor cells.

One such antigen is the "cytoplasmic melanoma-associated antigen" described by Natali et al. (1982). This molecule, identified by monoclonal antibody 465.12S, was found in the cytoplasm, on the cell surface, and shed into the medium of cultured human melanoma cells (Natali et al., 1982). Antibodies recognizing a similar antigen have been described by others (Bumol et al., 1982; T. F. Bumol, personal communication). Readily detectable levels of an antigen reactive with antibody 465.12S were also found in sera of patients with melanoma and other types of cancer (Giacomini et al., 1984). In the latter study, however, the antigen recognized in serum was not characterized, so it is not clear that the molecule detected was related to the antigen released by melanoma cells.

In our search for clinically useful serum markers for malignant disease, we identified a monoclonal antibody to an antigen secreted by lung carcinoma cells that was similar to the antigen described by Natali et al. (1982). Because of the potential usefulness of this antigen as a tumor marker for serum assays, we have characterized it in detail. In this paper, we demonstrate it to be structurally related to a component of normal human serum, which, to our knowledge, has not been described previously.

Materials and Methods

Materials. Culture media were from Gibco. Fetal bovine serum was from Gibco or Hyclone. Teflon-coated multiwell microscope slides (12 well) were from Meloy. Plastic culture dishes were from Corning or Costar. V8 protease and bovine serum albumin were from Sigma. Neuraminidase and Pansorbin (formalin-fixed *Staphylococcus aureus*) were from Calbiochem. Endoglycosidase H was from Miles. Nitrocellulose (BA85, 0.45 μm) was from Schleicher & Schuell. Protein A–Sepharose, CNBr-activated Sepharose 4B, Sephadex G-10, DEAE-Sephacel, and protein A conjugated Sepharose Cl-4B were from Pharmacia. NP-40 was from Particle Data Ltd. Poly(ethylene glycol), X-ray film, and the Kodavue staining kit were from Kodak. Acrylamide and the silver staining kit were from Bio-Rad. Prestained molecular weight markers were from BRL. ^{14}C-Methylated molecular weight markers, [^{3}H]glucosamine, [^{35}S]methionine, and ^{125}I were from Amersham. FITC[1]-conjugated goat anti-mouse immunoglobulin was from Tago. The Paragon serumprotein electrophoresis apparatus was from Beckman. Ampholines were from LKB. The L3 antibody was purified from ascitic fluid as described by Brown et al. (1981).

Cell Culture. Calu-1 lung carcinoma cells were obtained from the American Type Culture Collection. Cells were maintained in monolayer culture in growth medium [Dulbecco's modified Eagle medium (DMEM) containing 50 units of penicillin/mL, 50 μg of streptomycin/mL, and 10% (v/v) fetal calf serum (FCS)].

Indirect Immunofluorescence. Cells were collected by trypsinization and plated at a density of (5–20) × 10^3 per well (6 mm in diameter) on Teflon-coated multiwell slides (Meloy Laboratories). The medium was aspirated, and monolayers were washed once with phosphate-buffered saline (Gibco) containing 0.5 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and fixed in situ by the addition of 25 μL of a solution of paraformaldehyde (0.5% in PBS) for 20–30 min at 23 °C. Intracellular antigens were exposed by the addition of PBS containing 0.2% (v/v) NP40 for 5 min at 23 °C. Excess aldehyde groups were then blocked with binding buffer (Marquardt & Todaro, 1982) containing 15% FCS for a minimum of 15 min at 23 °C. Antibodies were applied at a concentration of 10 μg/mL in binding buffer. A fluoresceinyl isothiocyanate conjugated (FITC) secondary antibody was then added, and the slides were washed and air-dried. Slides were examined under a Leitz Orthoplan fluorescence microscope and photographed with Kodak Tri X Pan film.

Purification of L3 Antigen from Serum-Free Culture Medium. (*A*) *Preparation of Serum-Free Conditioned Medium.* Calu-1 cells were grown to confluence in roller bottles (850 cm^2) in the presence of growth medium. Monolayers were washed 3 times with serum-free growth medium and incubated with 150 mL of serum-free growth medium. After 3 days, the medium was collected, centrifuged to remove floating cells, and stored frozen at −70 °C until use.

(*B*) *Preparation of Concentrate.* Serum-free medium (790 mL) was concentrated to a volume of 17 mL by ultrafiltration (Amicon, PM10 membrane). The concentrate was removed, and the membrane was washed once with PBS (11 mL). The wash and concentrate were combined, and the mixture was centrifuged at 147000*g* for 30 min in a Ti 70 rotor (Beckman). The supernatant was found to contain most of the L3 antigenic activity and is hereafter referred to as the concentrate.

(*C*) *Immunoaffinity Chromatography.* The concentrate was passed over a column (1.5 mL) of Sepharose CL-4B to remove substances having affinity for Sepharose. The column was then washed with 3 mL of PBS. The flow-through was combined with the wash and added to a packed volume of 1 mL of L3 antibody conjugated Sepharose 4B. The mixture was held at 4 °C with rotation for a period of 18 h and then poured into a column made from a disposable hypodermic syringe. The flow-through was collected and the resin washed with PBS (10 mL) and solutions of 5 M NaCl (5 mL) and 0.02 M ammonium acetate (10 mL). L3 antigen was eluted from the column by the addition of a freshly made solution of triethylamine (75 mM). Since L3 antigenic activity was labile at elevated pH, the pH of the effluent was adjusted by collecting fractions (0.5 mL) in tubes containing 0.1 mL of 2 M ammonium acetate. Fractions containing L3 activity were identified with the competitive binding assay, pooled, and stored frozen at −20 °C.

Purification of L3 Antigen from Serum. (*A*) *Collection of Serum.* Blood was drawn and allowed to clot from 10 to 90 min at 23 °C. Samples were then stored at 4–6 °C from 30 min to 5 h. Serum was collected by centrifugation, aliquoted,

[1] Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; Endo H, endoglycosidase H; FITC, fluoresceinyl isothiocyanate; DMEM, Dulbecco's modified Eagle medium; FCS, fetal calf serum; SDS, sodium dodecyl sulfate; PBS, phosphate-buffered saline; EDTA, ethylenediaminetetraacetic acid.

and stored frozen at −70 °C until use. Levels of L3 antigenic activity did not differ significantly in serum or plasma from the same individual. Thawed aliquots were generally not refrozen since antigenic activity was found sensitive to repeated freeze–thawing. For purification of L3 antigenic activity, an aliquot of 10 mL of freshly frozen serum was thawed and clarified before use by centrifugation at 147000*g* for 60 min (Ti 70.1 rotor).

(*B*) *DEAE-Sephacel Chromatography.* A 10-mL column of DEAE-Sephacel was poured and equilibrated with PE buffer (0.02 M sodium phosphate, pH 7.12, and 0.005 M EDTA). Clarified serum was diluted to a volume of 130 mL with PE buffer before application to the ion exchange column. The column was washed with 10 column volumes of PE buffer, and antigenic activity was eluted with a 50-mL gradient of 0–0.5 M NaCl in PE buffer. Fractions containing L3 antigenic activity were identified by the competitive inhibition assay and pooled. L3 antigenic activity trailed slightly behind the bulk of eluted serum proteins.

(*C*) *Immunoaffinity Chromatography.* Pooled fractions (14 mL) from a DEAE-Sephacel column were subjected to immunoaffinity chromatography as described above.

Metabolic Labeling. Cells were collected by trypsinization. Viability, as determined by trypan blue exclusion, was usually greater than 95%. A total of 4×10^6 cells was collected by centrifugation and suspended in a volume of 1 mL of growth medium, lacking the amino acid methionine. [^{35}S]Methionine (800 Ci/mmol) was added to a final concentration of 0.5 mCi/mL, and cells were incubated with rotation at 37 °C for periods of 1–6 h. Labeled cells were then collected by centrifugation and washed prior to further manipulation.

Iodinations. Proteins were radiolabeled by the chloramine T procedure (Greenwood et al., 1963). Unreacted ^{125}I was removed by chromatography on a column of Sephadex G-10 equilibrated with PBS containing 1 mg/mL bovine serum albumin. The specific activity of labeled monoclonal antibodies used in this study was approximately 3×10^9 cpm/mmol. Samples containing L3 antigen were desalted on a spin column (Maniatis et al., 1982) of Sephadex G-10 prior to radiolabeling.

Immunoprecipitation Analysis. Metabolically labeled cells were collected by centrifugation and solubilized in RIPA buffer (Gentry et al., 1983) at a ratio of $(2–4) \times 10^6$ cells/mL. Following incubation for 10 min at 4 °C, the mixture was clarified by centrifugation (147000g for 30 min, Ti 70.1 rotor). Aliquots containing $(1–2) \times 10^7$ cpm (acid precipitable) of [^{35}S]methionine or ^{125}I were subjected to immunoprecipitation analysis as described by Brown et al. (1981). Monoclonal antibodies (1 μg) were bridged to formalin-fixed *S. aureus* with affinity-purified goat anti-mouse immunoglobulin (1–2 μg). When labeled culture medium was analyzed, an aliquot was used that was equivalent to the percentage of the total cell extract analyzed. Precipitates were washed 3–4 times in TNEN buffer (Brown et al., 1981) and stored frozen at −20 °C until use.

Electrophoresis. (*A*) *SDS–Polyacrylamide Gel Electrophoresis (SDS–PAGE).* SDS–PAGE was run according to Laemmli (1970). The resolving gel (0.75 mm) was a 10–20% linear acrylamide gradient, and the stacking gel contained 5% acrylamide. Samples were boiled in sample buffer containing 5% 2-mercaptoethanol, and the immunoabsorbent was removed by centrifugation prior to electrophoresis. The standard proteins (molecular weight) used were myosin (200 000), phosphorylase *b* (92 400), bovine serum albumin (69 000), ovalbumin (46 000), chymotrypsinogen (26 000), β-lactoglobulin (18 400), lysozyme (14 300), and cytochrome *c* (12 300).

(*B*) *Paragon Electrophoresis.* Serum protein electrophoresis was conducted with preformed agarose gels on a Paragon apparatus (Beckman) according to the manufacturers instructions.

Immunoblotting. Proteins to be analyzed were separated by either SDS–PAGE or Paragon. Samples separated by SDS–PAGE were electrophoretically transferred to nitrocellulose as described by Burnette (1981) at 5 V/cm for a period of approximately 18 h at 4 °C. Samples separated by Paragon were transferred to nitrocellulose by overlaying the electrophoretogram with a sheet of nitrocellulose paper. Nitrocellulose was hydrated in the transfer buffer of Burnette (1981) without SDS before use. A stack of paper towels approximately 1-in. thick was placed over the nitrocellulose and weighted down with a glass plate and reagent bottle. Transfer was complete within a 1-h period, as judged by staining of the gel following transfer. Nitrocellulose blots were blocked by a 1-h incubation in Blotto (Johnson et al., 1984). L3 antigen was revealed by incubation of the blots with $(1–4) \times 10^6$ cpm/mL ^{125}I-L3 antibody in Blotto for 1 h at 23 °C. Blots were washed extensively with Blotto and dried before autoradiographic exposure.

Binding of ^{125}I-L3 Antibody. Calu-1 cells $[(4–10) \times 10^4]$ were plated in 48-well cell culture dishes and processed as for immunofluorescence. Various concentrations of ^{125}I-labeled antibody were added in a final volume of 0.1 mL for a period of 60 min at 23 °C. Monolayers were then washed extensively with binding buffer and solubilized by the addition of 0.5 M NaOH. Cell-bound radioactivity was determined on a γ counter. Samples were assayed in duplicate; duplicate samples generally varied by less than 20%. To measure nonspecific antibody binding, identical wells were incubated with a 50–100-fold excess of unlabeled L3 antibody, which usually reduced binding of ^{125}I-labeled antibody by more than 90%.

Competitive Binding Assay. L3 antigenic activity was measured by determining the ability of the antigen to inhibit binding of ^{125}I-labeled L3 antibody to formalin-fixed monolayers of Calu-1 cells. ^{125}I-L3 antibody was added to cells at a concentration of 0.4 μg/mL in the presence or absence of solutions containing L3 antigenic activity. The final assay volume was 0.1 mL. Under these conditions, the binding of ^{125}I-L3 antibody was more than 95% specific, as judged by the ability of unlabeled L3 antibody to compete for binding. Various dilutions of the test solutions were added, and the binding of ^{125}I-labeled antibody was measured as described above. One unit of L3 antigenic activity is defined as the amount necessary to inhibit the binding by 50% of ^{125}I-L3 antibody added at 0.4 μg/mL.

Glycosidase Treatments. Immunoprecipitates of [^{35}S]-methionine or ^{125}I-labeled L3 antigen were prepared as described. The pelleted immunoabsorbant was treated with endoglycosidase H (Endo H) as described by Omary and Trowbridge (1981) or by neuraminidase as described by the supplier. Reactions were stopped with concentrated SDS–PAGE sample buffer.

Preparation of L3–Sepharose. CNBr-activated Sepharose was derivatized according to the manufacturer's instructions. Efficiency of coupling was assessed by measuring absorbance at 280 nm of the antibody solution before and after coupling and was determined to be approximately 10 mg of L3 antibody/mL of packed resin.

Amino-Terminal Sequencing by Edman Degradation. Immunoaffinity-purified L3 antigen was subjected to preparative

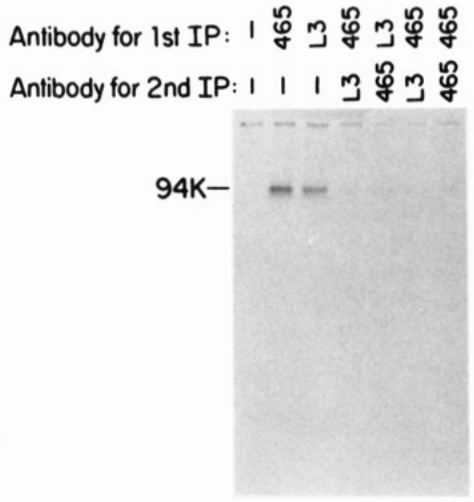

FIGURE 1: Sequential immunoprecipitation analysis of L3 antigen with an antibody recognizing a melanoma antigen. Culture medium from Calu-1 cells that had been metabolically labeled with [^{35}S]-methionine was subjected to immunoprecipitation analysis with the indicated monoclonal antibody (1st IP). The supernatant from the first IP was then treated with the indicated antibodies during the 2nd IP. Antibody 465.12S (labeled 465) was provided by S. Ferrone during the *First International Workshop on Melanoma Associated Antigens*.

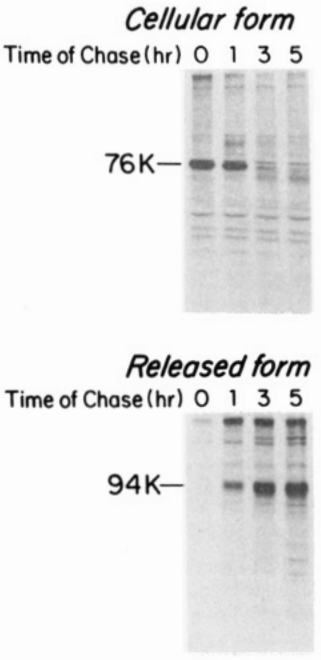

FIGURE 2: Pulse–chase analysis of L3 antigen. Calu-1 cells were metabolically labeled with [^{35}S]methionine in methionine-free growth medium for 1 h at 37 °C, collected by centrifugation, and resuspended in growth medium containing the normal amount of unlabeled methionine. After the indicated time of chase (at 37 °C), aliquots were removed and the cells collected by centrifugation and extracted as described under Materials and Methods. Aliquots representing equivalent proportions of the cell pellet and medium were subjected to immunoprecipitation as described. For simplicity, control samples to which no L3 antibody was added have been omitted from the figure. Control samples do not exhibit the major M_r 76 000 and M_r 94 000 proteins shown above but do exhibit equivalent amounts of the other (background) bands.

SDS–PAGE. The gel was stained, and the L3 antigen was excised and immediately subjected to electroelution and electrodialysis (Hunkapiller et al., 1983). Automated Edman degradation was performed with approximately 100 pmol of conditioned medium-derived L3 antigen (based on the yield of identified Met-6) and 38 pmol of serum-derived L3 antigen (based on the yield of identified Val-1) in a gas-phase sequencer, (Model 470A, Applied Biosystems, Inc., Foster City, CA). The phenylthiohydantoin amino acids were analyzed by reverse-phase HPLC.

Results

Selection of L3 Antibody. To identify monoclonal antibodies that react with antigens shed or secreted by cultured lung tumor cells, we screened a panel of antibodies with specificity for nonsmall cell lung cancer (I. Hellström et al., unpublished results) for the ability to immunoprecipitate antigens released into the culture medium by Calu-1 lung carcinoma cells. Culture medium from cells metabolically labeled with [^{35}S]-methionine or [^{3}H]glucosamine was subjected to immunoprecipitation analysis and SDS–PAGE as described under Materials and Methods. L3, a monoclonal antibody of the IgG1 isotype, was found to precipitate a glycoprotein antigen of M_r 94 000 from culture medium (illustrated in Figure 1), indicating that this antigen is shed or secreted by Calu-1 cells. A detailed description of the histological distribution of the antigen(s) detected by the L3 antibody will be described elsewhere (I. Hellström et al., unpublished results). Briefly, the L3 antibody reacted with essentially all lung tumors tested and with most colon and breast carcinomas. It also reacted to a weaker extent with some normal tissues, particularly lung and kidney.

The L3 Antigen Is Identical with Antigen Recognized by Antibody 465.12S. The L3 antigen was expressed by cell lines derived from a number of different tumor types (data not shown). It was conceivable, therefore, that it had been described previously as an antigenic component of a tumor type other than carcinoma of the lung. To test this possibility, we performed a computer-assisted literature search for shed or secreted tumor-associated antigens. We discovered several reports (Natali et al., 1982; Bumol et al., 1982; Wilson et al., 1981) of melanoma-secreted protein antigens that were of similar size as the L3 antigen. Small amounts of one of the previously described monoclonal antibodies specific for this antigen (465.12S; Natali et al., 1982) was available to us through our participation in the *First International Workshop on Melanoma Associated Antigens* (Hellström et al., 1982). We employed this antibody in a sequential immunoprecipitation experiment to test for identity between the L3 antigen and the antigen recognized by antibody 465.12S. The results of this experiment are shown in Figure 1. Both antibodies specifically precipitated a M_r 94 000 protein species from culture medium of cells metabolically labeled with [^{35}S]-methionine (lanes 2 and 3). Depletion of the culture medium for either L3 or 465.12S antigen by immunoprecipitation resulted in a concomitant depletion of antigenic activity of the alternative antigen (lanes 3 and 4). This shows that the antigenic determinants recognized by both antibodies are carried on the same molecule. However, the epitopes recognized by the two antibodies are probably different since we were unable to detect competition by antibody 465.12S for binding of ^{125}I-L3 antibody (data not shown).

Biosynthesis of L3 Antigen. Various forms of the antigen recognized by antibody 465.12S were identified by Natali et al. (1982). To biochemically characterize the L3 antigen, we first studied its biosynthesis. Our initial results had indicated that the L3 antigen found in culture medium was a glycoprotein of M_r 94 000. When cell extracts metabolically labeled with [^{3}H]glucosamine, [^{35}S]methionine, or [^{35}S]cysteine were used, a major glycoprotein antigen of M_r 76 000 was precipitated by the L3 antibody (illustrated in Figure 2). Variable amounts of a M_r 94 000 labeled species were also seen occa-

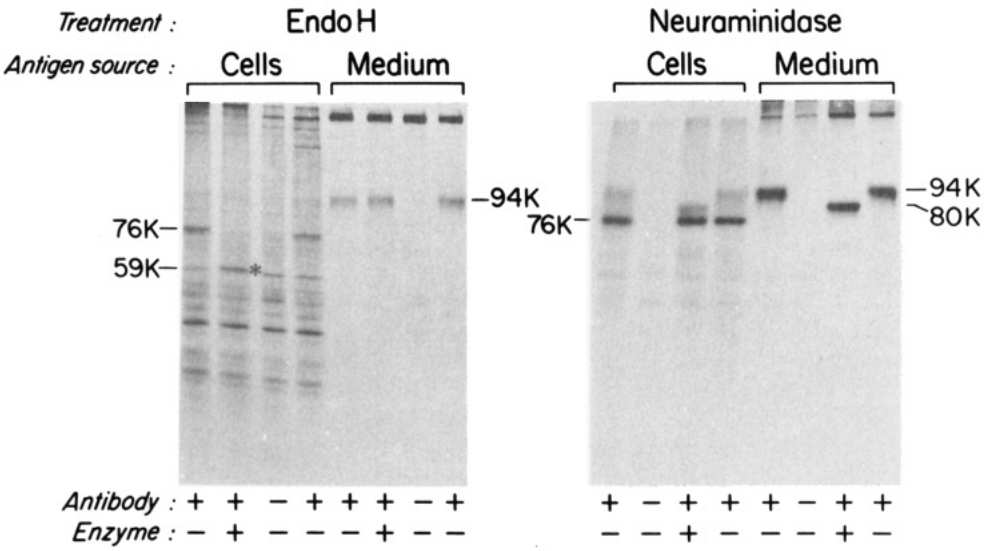

FIGURE 3: Glycosidase sensitivity of immunoprecipitated L3 antigen. Immunoprecipitates from Calu-1 cell extracts or culture medium labeled with [^{35}S]methionine were made with or without the L3 antibody and were treated with the indicated glycosidase as described under Materials and Methods. Samples from the left panel were labeled for 6 h with [^{35}S]methionine, while those from the right panel were labeled for 1 h, washed, and placed into unlabeled methionine-containing medium for an additional 2 h. Variations in background band intensities and the amounts of the M_r 94000 form associated with the cell pellet presumably reflect difference in labeling conditions. One sample in each experiment (the left-most sample from each antigen source) was incubated at 37 sC in the absence of enzyme; one sample (the right-most sample for each source) was stored frozen after immunoprecipitation. The asterisk (*) indicates the fragment of the cellular form of the L3 antigen formed by Endo H treatment; this fragment can be distinguished more clearly from background band on the original X-ray film.

sionally. The latter protein was identical in mobility with the antigen precipitated from culture medium. When L3 antibody was omitted from the immunoprecipitation reactions, the amounts of both the M_r 76000 and the M_r 94000 species precipitated were significantly reduced. Mobilities of both forms were not affected when SDS–PAGE was run under nonreducing conditions. These results indicate that the L3 antibody recognizes both cell-associated (M_r 76000) and released (M_r 94000) glycoproteins.

In order to determine the relationship between the forms of the L3 antigen, we radiolabeled cells with [^{35}S]methionine and performed a pulse–chase experiment as presented in Figure 2. Cells were pulse-labeled for 1 h with [^{35}S]methionine, placed into medium containing unlabeled methionine for various times, and subjected to immunoprecipitation analysis. Initially, all specifically immunoprecipitable cell-associated radioactivity was found in an M_r 76000 protein. (The other labeled proteins seen were also found in samples from which antibody was omitted.) During the chase period, radioactivity in the M_r 76000 form decreased while radioactivity found in the form released into the medium (M_r 94000) increased. Radioactivity in the cell-associated form disappeared with the same kinetics as radioactivity in the released form appeared; the $t_{1/2}$ for both processes was determined by densitometric analysis to be approximately 1.5 h. These results suggest that the M_r 76000 form of the antigen is a precursor of the M_r 94000 form.

Carbohydrate Composition of L3 Antigen. Since the L3 antigen is a glycoprotein, carbohydrate modifications might conceivably account for the apparent increase in size of the L3 antigen observed during its release from the cell. We examined this possibility by treating immunoprecipitates containing the cellular and released forms of the molecule with glycosidases of known specificity. As shown in Figure 3, the size of the cellular form (M_r 76000) of the L3 antigen was reduced to M_r 59000 by treatment with endoglycosidase H (Endo H) but was unaffected by neuraminidase, indicating that it contained N-linked high-mannose oligosaccharides (Tarentino & Maley, 1974). This result is consistent with our finding (data not shown) that maturation is sensitive to tunicamycin, a known inhibitor of N-linked glycosylation (Lehle & Tanner, 1976). In contrast, the released (M_r 94000) form of the antigen was Endo H resistant, but was reduced in size to M_r 80000 by neuraminidase treatment, indicating the presence of terminal sialic acid residues on the released form of the L3 antigen. Thus, the conversion of L3 antigen from a cell-associated to a released form is accompanied by maturation of N-linked carbohydrate side chains.

L3 Antigen Has a Subcellular Distribution Expected for a Secreted Protein. Biochemical data indicated that L3 antigen was shed or secreted into the medium of cultured cells. To better understand the mechanism of antigen release, the subcellular localization expression of the L3 antigen was determined by indirect immunofluorescence on the lung carcinoma cell line Calu-1 (Figure 4). No fluorescence was observed when intact formalin-fixed cells were used. This was consistent with the finding that no detectable binding of L3 antibody was observed when viable cells were stained and examined with the fluorescence-activated cell sorter (data not shown). In contrast, when formalin-fixed cells were permeabilized with a nonionic detergent prior to antibody addition, a distinctive, immunologically specific staining pattern was observed (compare panels B and C of Figure 4). The pattern was stipled in appearance and tended to be localized in a perinuclear fashion, as has been demonstrated for antigens localized in the Golgi complex (Louvard et al., 1982). Similar staining patterns were observed with a number of human tumor and normal cell lines. This staining pattern indicates that the cell-associated (M_r 76000) form of the L3 antigen is localized intracellularly, probably in the Golgi apparatus. Since a plasma membrane associated form of the antigen was not detected, release of the M_r 94000 form from the cell most likely occurs by secretion rather than shedding (Natali et al., 1982).

L3 Antigen Is a Component of Normal Serum. These findings suggested that the L3 antigen might also be released into the blood stream by tumor cells in vivo. To investigate this possibility, we used an immunoblotting technique to detect

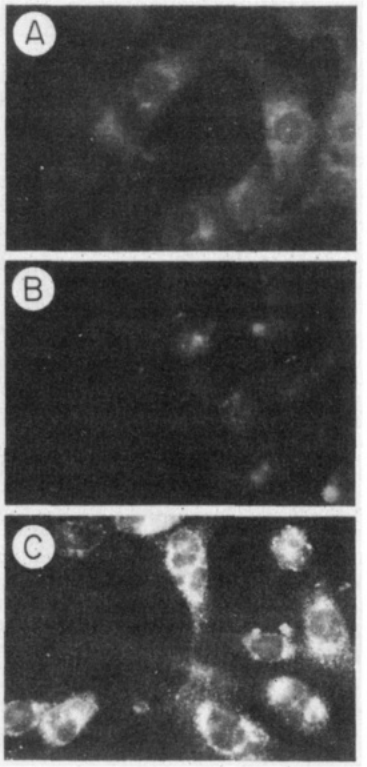

FIGURE 4: Subcellular localization of L3 antigen. Calu-1 cells were grown on glass slides and processed for indirect immunofluorescence as desribed under Materials and Methods. (Panel A) Cells were labeled with L3 antibody, followed by an FITC-conjugated goat anti-mouse Ig antiserum. (Panel B) Cells were permeabilized by treatment with a nonionic detergent as described under Materials and Methods, prior to labeling with FITC-conjugated secondary antibody; L3 antibody was not added to these cells. (Panel C) Cells were permeabilized prior to labeling with L3 antibody and, subsequently, a FITC-conjugated secondary antibody. Final magnification is approximately 550×.

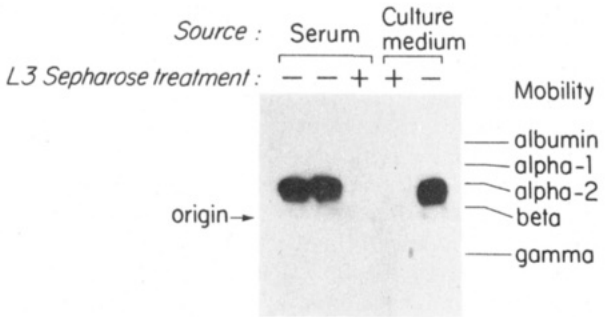

FIGURE 5: Immunoblot analysis of L3 antigen from normal human sera and calu-1 cultured medium. Aliquots of the indicated samples were applied to a preformed agarose electrophoresis gel (Paragon SPE) as described by the manufacturer (Beckman). The gel was run at 100 V for 30 min in Paragon B2 barbital buffer, pH 8.6 (Beckman). Following electrophoresis, the resolved proteins were transferred to nitrocellulose and subjected to immunoblot analysis as described under Materials and Methods. Serum samples were from two healthy male subjects; 1 μL of serum was diluted 5-fold with B2 buffer prior to application. Culture medium was concentrated approximately 7-fold by ultrafiltration before use; aliquots of approximately 3 μg of protein in a volume of 5 μL were applied. Where indicated, samples were subjected to immunoaffinity chromatography on L3–Sepharose prior to electrophoresis. Mobilities of the following proteins were determined by protein staining or by using tracer amounts of ^{125}I-labeled material: human serum albumin (albumin), α-1 antitrypsin (alpha-1), α-2 macroglobulin (alpha-2), transferrin (beta), and human immunoglobulin (gamma).

immunoreactive molecules in serum or plasma from normal individuals and patients with advanced lung cancer. Concentrated culture medium from Calu-1 cells and fresh human

Table I: Purification of L3 Antigenic Activity from Culture Medium of Calu-1 Cells

sample	vol (mL)	protein (mg)[a]	act. (units)[b]	yield (%)	sp act. (units/mg)	purification factor
medium	910	35.5	18 600	100	522	1
concentrate	27.8	11.9	12 900	70	1 090	2.08
L3 affinity column	4.2	0.139	6 470	35	46 500	89.1

[a] Determined by the method of Bradford. [b] Amount required to inhibit binding of ^{125}I-labeled L3 antibody by 50% in the competitive binding assay described under Materials and Methods.

Table II: Purification of L3 Antigenic Activity from Normal Human Serum

sample	vol (mL)	protein (mg)[a]	act. (units)[b]	yield (%)	sp act. (units/mg)	purification factor
serum	10	880	11 800	100	13.3	1
DEAE pool	14	118	9 040	79	79	5.9
L3 affinity column	10	0.1	4 550	39	45 500	3420

[a] Determined by the method of Bradford (1976). [b] Amount required to inhibit binding to ^{125}I-labeled L3 antibody by 50% in the competitive binding assay described under Materials and Methods.

serum was analyzed by electrophoresis under nondenaturing conditions on a standard commercial system (Beckman Paragon) for serum protein analysis. The resolved proteins were then transferred to nitrocellulose and analyzed for ^{125}I-L3 antibody binding as described under Materials and Methods. Concentrated culture medium from Calu-1 cells and all serum samples tested from both normal individuals (Figure 5) and tumor patients (data not shown) revealed a single immunoreactive component(s) with α-2 mobility. The reactive species could be removed by passage of the serum or concentrated culture medium over an L3 affinity column. These results indicated that the L3 antibody recognized a component of human serum. To fully evaluate the diagnostic potential of the antibody, characterization of its antigen from normal serum and tumor cell conditioned medium was necessary.

Purification of L3 Antigen from Culture Medium and Human Serum. Precise molecular characterization of the L3 antigen was not possible without its prior purification. High-resolution immunoblotting techniques could not be used since immunoreactivity was found to be sensitive to the denaturing conditions employed in SDS–PAGE. L3 antigenic activity was therefore purified from both serum-free conditioned medium and normal human serum, by procedures that are described under Materials and Methods, and summaries of the procedures are presented in Tables I and II. SDS–PAGE analysis of the purified material is presented in Figure 6A, and an analysis of the immunoreactivity of purified preparations is presented in Figure 5B.

Antigenic activity was purified from culture medium approximately 90-fold with an overall yield of 35% (Table I). Analysis of the purified preparation by SDS–PAGE (Figure 6A, lane 2) revealed a major component of M_r 94 000 (form I) and a minor component of M_r 76 000 (form II). A prominent band that comigrated with the form I species was observed in the starting material (Figure 6A, lane 1). When the purified preparation was subjected to immunoblotting analysis (Figure 6B, lane 1), both the form I and form II components were found to be immunoreactive. When the preparation was first radiolabeled with ^{125}I and then immunoprecipitated with L3 antibody, both form I and form II components were specifically precipitated (Figure 6B, lanes 3 and 4), together with

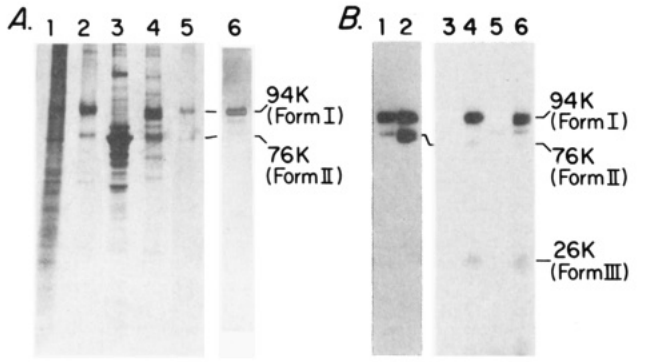

FIGURE 6: Purification of L3 antigen. (A) Silver stain analysis of purification steps. The indicated samples were taken from steps in the purificatiyon of the L3 antigen, as described in the text and under Materials and Methods, subjected to electrophoresis, and stained with a commercial silver (Bio-Rad) or nickel (Kodak, lane 6) staining kits. The sample in lane 6 has been overdeveloped to show more clearly the minor components. (Lane 1) An aliquot (50 μg of total protein) of the concentrated serum-free conditioned medium from Calu-1 cells. (Lane 2) Immunoaffinity purified L3 antigen from conditioned medium, 1 μg of protein. (Lane 3) Normal human serum, 4 μg of protein. (Lane 4) Immunoaffinity-purified L3 antigen from normal human serum 0.7 μg of protein. (Lane 5) L3 antigen from culture medium purified by preparative SDS–PAGE. (Lane 6) L3 antigen from serum purified by preparative SDS–PAGE. (B) Immunoreactivity of purified L3 antigen. The indicated samples were tested for immunoreactivity by immunoblotting after SDS–PAGE (lane 1 and 2) or by radioiodination and subsequent immunoprecipitation (lanes 3–6). Approximately 1×10^7 cpm of ^{125}I-labeled L3 antigen, prepared as described under Materials and Methods, was subjected to analysis. (Lane 1) Immunoaffinity-purified L3 antigen from conditioned medium, 0.5 μg. (Lane 2) Immunoaffinity-purified L3 antigen from serum, 3 μg. (Lane 3) ^{125}I-L3 antigen from conditioned medium, immunoprecipitated without antibody. (Lane 4) Same as in lane 3, with L3 antibody. (Lane 5) ^{125}I-L3 antigen from serum, immunoprecipitated minus L3 antibody. (Lane 6) Same as in lane 5, plus L3 antibody.

a radiolabeled component of M_r 26 000 (form III) that was not clearly visible after silver staining or immunoblotting.

To purify L3 antigen from serum, an initial step of ion exchange chromatography was employed prior to immunoaffinity chromatography to prevent overloading the affinity column with protein. L3 antigen was purified more than 3000-fold from normal human serum with a yield of 39% (Table II). Analysis of the purified material by SDS–PAGE revealed the major components to be proteins that comigrated with the form I and form II components purified from culture medium (Figure 6A, lane 4); neither form was a major component of the initial serum sample (lane 3). It is noteworthy that both form I and form II antigens from serum were resolved into two distinct components on the original gel, possibly indicating microheterogeneity due to glycosylation differences. both forms were reactive following immunoblotting analysis (Figure 6B, lane 2) and after radioiodination and immunoprecipitation (lanes 5 and 6). As with the antigen purified from culture medium, an additional form of M_r 26 000 (form III) was detected after immunoprecipitation (lanes 4 and 6).

These results indicate that L3 antigenic activity is expressed on two components (forms I and II) found in both culture medium and serum. Further purification of the form I molecule from both sources by preparative SDS–PAGE (Figure 6A, lanes 5 and 6) resulted in preparations that contained predominantly form I molecules, although even after this procedure form II molecules were sometimes observed. The amount of form II molecules in SDS–PAGE-purified preparations was variable.

Comparison of L3 Antigen from Culture Medium and Human Serum. The observation that the L3 immunoreactive

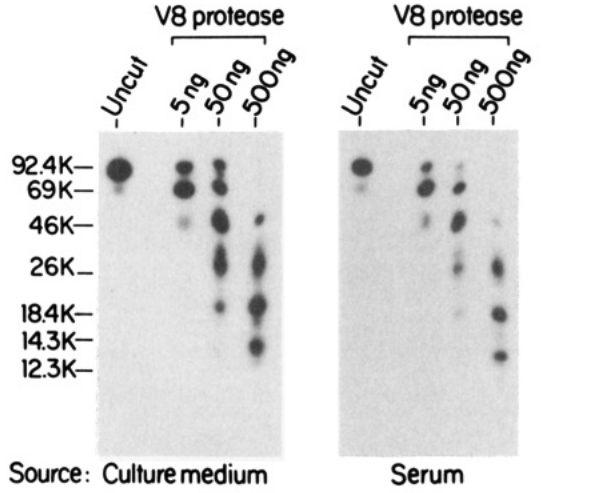

FIGURE 7: V8 fingerprint analysis of form I L3 antigen from culture medium and serum. L3 antigen was purified from culture medium or serum, radioiodinated, and subjected to immunoprecipitation analysis and preparative SDS–PAGE as described under Materials and Methods. ^{125}I-Labeled form I L3 antigen was localized in the wet gel by autoradiography, excised, and fingerprinted with the indicated concentration of V8 protease as described by Cleveland et al. (1977).

species found in serum comigrated with the antigens found in culture medium suggested that these molecules were structurally related. We further compared ^{125}I-labeled and immunoprecipitated antigens purified from both sources by two-dimensional isoelectric focusing–SDS–PAGE (data not shown). The two-dimensional mobilities of both antigens were closely similar, with both form I and form II components migrating as heterogeneous species in the pH range of 4.2–5.0. Form I antigen from both sources also revealed two minor components migrating at approximate pH values of 5.8 and 6.2.

The carbohydrate composition of both antigens was also examined by testing for glycosidase sensitivity. Form I and form II L3 antigens from both sources were found to be Endo H resistant, while neuraminidase treatment of both resulted in the appearance of the characteritic M_r 80 000 fragment seen following treatment of metabolically labeled antigen (see also Figure 3). The neuraminidase sensitivity of form II antigen indicates that it is different from the M_r 76 000 cell-associated form of the L3 antigen (Figure 2).

To confirm that both molecules were structurally similar, we subjected form I molecules from both sources to a series of partial protease cleavage reactions as described by Cleveland et al. (1977). The radiolabeled form I antigens (M_r 94 000) purified from both culture medium and serum were excised from a preparative SDS–PAGE gel and subjected to fingerprinting with V8 protease (Figure 7). Both molecules yielded identical partial cleavage patterns; at least five distinctive partial cleavage products were observed (M_r 76 000, 49 000, 31 000, 20 000, and 14 300) in both cases. These results demonstrate that L3 antigens purified from culture medium and human serum are highly related.

We next investigated the relationship between the three molecular weight forms of L3 antigen from serum. The overall fingerprints for forms I and II were quite similar, although the form II molecule did not yield the M_r 31 000 and M_r 14 300 fragments derived from the form I molecule (data not shown). The patterns suggested that form I and form II molecules were closely related. The form III antigen shared a M_r 14 300 partial cleavage product with the larger antigenic forms found in serum, which may indicate that the form III antigen was

Table III: Amino-Terminal Sequence of L3 Antigen from Two Sources[a]

Culture Medium	
V^{1}-N-D-G-D^{5}-M-R-L-A-D^{10}-G-G-A-T-N^{15}-Q-G-R-V-E^{20}-I-F-Y-R-G^{25}-Q-W-G-T-V^{30}	
Normal Human Serum	
V^{1}-N-D-G-D^{5}-M(R)L-A-D^{10}-G(G)A-T-N^{15}-Q	

[a] N-Terminal sequences were determined by automated Edman degradation as described under Materials and Methods. Parentheses indicate tentative assignments.

also structurally related to form I antigen. the close similarities in structure between the various forms suggest that form II and III molecules are derived from form I antigen, probably by proteolytic degradation during the isolation procedure. This possibility is consistent with the finding that form II and III molecules are not seen in immunoprecipitates prepared from culture medium (Figures 1–3).

Amino-Terminal Sequence of L3 Antigen from Culture Medium and Serum. To determine if L3 antigen was related to any of the many well characterized serum proteins, we determined the amino-terminal sequences of form I molecules from both culture medium and serum. Three preparations of the antigen from culture medium were subjected to sequence determination. One of these preparations contained no detectable form II molecules. A single preparation of the antigen from serum was also analyzed; in this case, trace amounts of form II antigen and an unidentified M_r 80000 component were present (see Figure 6A, lane 6). A single amino acid sequence was found in all preparations. The N-terminal sequences of form I L3 antigens were aligned in Table III. Both sequences were identical through the first 16 residues.

Comparison of the 30 N-terminal amino acids of the L3 antigen with protein sequences present in the National Biomedical Research Foundation protein bank (November 1985 edition) revealed no significant homologies. The sequence of the L3 antigen was also compared with that of several serum proteins not currently listed in this data base, including human C1 esterase inhibitor (Harrison, 1983) and human, α-2-thiolproteinase inhibitor (Ohkubo et al., 1984). None of these sequences revealed any significant homologies with the N-terminal sequence of the L3 antigen.

Discussion

The antigen recognized by antibodies L3 and 465.12S has the property of being readily released into the medium of cultured cells, suggesting that it might be useful as a serum diganostic marker. For this reason, we have characterized the antigen in detail. Several aspects of this work are of significance.

We have structurally characterized a commonly identified "tumor-associated antigen". This was important because the data we have obtained can be used as the basis for future comparisons. Since sequence data can provide important insights into protein function [for example, see Brown et al. (1982)], our data may help elucidate the biological relevance of this antigen. Our data also differ somewhat from previously published results (Natali et al., 1982; Bumol et al., 1982). The antigen recognized by antibody 465.12S was described as a "cytoplasmic melanoma-associated antigen", expressed on the cell surface and shed by tumor cells. Our results suggest that the claim that the antigen is melanoma-associated are misleading. Since the L3 antibody was raised against lung tumors, the molecule is likely to be an antigen component of many human tumors. The antigen is also a component of normal serum. Since our immunofluorescence and binding data indicate that the cell-associated form of the L3 antigen is localized within the cell rather than on the cell surface, release of the antigen is most likely due to protein secretion rather than shedding from the cell surface. Our observations thus indicate that the L3 antigen is a widely expressed and secreted glycoprotein.

Table IV: Characteristics of L3 Antigen in Normal Human Serum

concn (μg/mL)	5.8 ± 2.6[a]
M_r	94000 (form I)
	76000 (form II)
	26000 (form III)
mobility	α-2
p*I*	4.2–5.0 (forms I and II)
N-terminal amino acid	Val (form I)
type of carbohydrate	complex N-linked

[a] Determined as the mean value (± SD) from seven normal healthy individuals. Sera were tested at a final concentration of 5.7% by volume of the competitive binding assay, with immunoaffinity purified L3 antigen from culture medium as a standard. Values were corrected for the purity of the standard preparation, which was estimated (by densitometric scanning of a silver stained SDS–PAGE gel) to be 66%.

We have also shown that the L3 antibody recognizes a novel serum protein whose properties are summarized in Table IV. Comparison of these properties with those of known serum proteins (Putnam, 1984) indicates that the L3 antigen is not identical with any previously characterized serum protein. A serum component having properties most closely resembling the L3 antigen is C1 esterase inhibitor, a glycoprotein of M_r 100000 having α-2-mobility. However, the N-terminal sequence we have determined for the L3 antigen does not show homology with that determined for C1 esterase inhibitor (Harrison et al., 1983), and the two-dimensional electrophoretic mobilities of these two proteins differ (data not shown). Likewise, depletion of serum of L3 antigen by immunoaffinity chromatography did not affect reactivity of the serum with an antiserum (Alantic Antibodies) to C1 esterase inhibitor (data not shown). Taken together, these observations indicate that the L3 antigen is distinct from C1 esterase inhibitor. α-2-Thioproteinase inhibitor also exhibits α-2 mobility and is similar to form I L3 antigen in molecular weight and p*I* (Putnam, 1984) but does not share sequence homology (Ohkubo et al., 1984). The L3 antigen also does not readily correspond to any of a list of serum proteins of unknown functions identified by two-dimensional electrophoretic analysis (Anderson et al., 1984). Since we were unable to identify form I L3 antigen as a known serum protein, we conclude that it represents a previously uncharacterized serum component. It is possible, however, since the structurally related form II and III antigens were not fully characterized, that one of these forms may be homologous to a known serum protein.

Finally, our results have important implications for the use of the L3 antibody for serum diagnostic tests. Giacomini et al. (1984) have demonstrated that detectable serum levels of an antigen recognized by antibody 465.12S could be detected in sera from cancer patients. They could not, however, consistently correlate serum antigen levels with extent of disease. These results can be explained by our demonstration that the L3 antigen is a component of normal serum. Since normal serum levels of the antigen are relatively high, it is unlikely that small tumors would actually release enough of the antigen to cause an elevation in serum levels. We have demonstrated elevated serum levels of this antigen in some patients with malignant disease but have also found elevated levels in patients with various nonmalignant diseases (data not shown). It is possible that a systematic evaluation of serum levels of the L3 antigen may indicate a useful correlation with some

pathologic condition. However, since the serum form of the L3 antigen and the form produced by cultured tumor cells are structurally indistinguishable, it seems likely that any differences will be quantitative rather than qualitative in nature.

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