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Research Article

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A B S T R A C T   Studies of a number of properties of the pathological γA-proteins in the first four cases of the recently recognized alpha-chain disease demonstrate that, as in γ-heavy-chain disease, the abnormal protein is devoid of light chains and represents a portion of the α-heavy chain related to the Fc-fragment. In two patients, serum electrophoresis showed a broad abnormal band, whereas in the two others the pathological protein was not noticeable on the electrophoretic pattern. The diagnosis of α-chain disease can be established without purification of the protein by immunoelectrophoresis and gel diffusion experiments using selected antisera to γA and a reference α-chain disease protein. All four proteins belonged to the α1-subclass, displayed electrophoretic heterogeneity, and showed a strong tendency to polymerize. The polymers occurred in vivo and were held together both by disulfide bonds and by strong noncovalent forces. Two of the three purified proteins had a very high carbohydrate content. The abnormal protein was always found in concentrated urines in variable but generally low amounts. It was not detected in parotid saliva but was present in significant amounts in jejunal fluid of all four patients. The α-chain disease protein was shown to be associated with the secretory piece in external secretions of two patients.

The clinicopathological features were strikingly similar in the four patients. All patients were affected with a neoplastic and mostly plasmacytic proliferation involving primarily the whole length of the small intestine and the mesenteric nodes and all exhibited a severe malabsorption syndrome. While Israeli authors have emphasized the frequency of this type of abdominal lymphoma in young Arabs and non-Ashkenazi Jews, two of our patients were Kabyles, one a Syrian Arab, and one an Eurasian. Cellular studies showed that the pathological protein was synthesized by the proliferating cells in the lymphoid tissue of the digestive tract and in the mesenteric nodes, and that there was no detectable light-chain synthesis at the intracellular level.

INTRODUCTION

When Franklin reported the first case of γ-heavy-chain disease, an uncommon form of malignant lymphoma characterized by the presence in serum and urine of a naturally occurring immunoglobulin representing mainly the Fc-fragment of the γ-chain, he anticipated that similar conditions involving α- and μ-chains would ultimately be identified (1, 2). In fact, a new type of immunoglobulin abnormality, characterized by the presence in serum and urine of a protein devoid of light chains and closely related to the heavy polypeptide chains of γA1-globulin, has recently been described in this laboratory (3). In view of the similarity with γ-heavy-chain disease, the designation "alpha chain disease" was applied to the corresponding syndrome.

The first recognized patient was a young Arab female affected with a malignant lymphoma involving the whole length of the small intestine; she exhibited a severe malabsorption syndrome (4). This type of lymphoma has been noted to be frequent in young Arabs and non-Ashkenazi Jews by several Israeli authors (5, 6). During the past several months, we have detected among patients from Paris hospitals with similar clinicopathological features three new cases with an analogous immunoglobulin A abnormality. Thus alpha chain disease probably represents a true entity defined by characteristic clinical, biological, and pathological features. The case histories of these three additional patients will be published in detail elsewhere (7-9). This report presents some immunological and physicochemical properties of the pathological immunoglobulin in these four cases of alpha chain disease, as well as some data on cellular synthesis.

METHODS

Collection and preservation of biological fluids. Some blood samples from the two last patients were collected in iodoacetamide at a final concentration of 2 mg/ml to suppress disulfide interchange. 24-hr urine samples were collected in 200 mg iodoacetamide and 1 g sodium azide. They were filtered and concentrated by negative pressure dialysis in VisKing 23/32 tubing in the presence of iodoacetamide. Jepunal fluid was collected in a preservative mixture (an aqueous solution containing 131 mg/ml of ε-aminocaproic acid, 10 mg sodium azide, 1 mg kanamycin, 20 mg iodoacetamide, and 10 mg of soybean trypsin inhibitor/ml in a ratio of 9:1, v/v). Immediately after collection, jejunal fluid was heated at 56°C for 30 min. After filtration and centrifugation, it was concentrated by negative pressure dialysis against the preservative mixture diluted 10 times in buffered saline. Parotid saliva was collected with a special plastic cup[1] and was concentrated by negative pressure dialysis in VisKing 23/32 membranes. All samples were stored frozen at −20°C.

Immunological studies

Antisera were prepared in rabbits by immunization with antigens in complete Freund's adjuvant. 20 antisera to several purified γA-myeloma proteins were made specific by suitable absorption with serum of patients having selective γA deficiency and, if necessary, light chains, γG, or agammaglobulinemic serum. Several control experiments were performed in order to ascertain that these absorbed antisera reacted only with γA. They were then tested against several γA-myeloma proteins of known light- and heavy-chains subclasses in order to select: (a) antisera demonstrating antigenic deficiency of the γA2-proteins (10–12) and (b) antisera containing antibodies which precipitate only when κ- or λ-chains are combined to α-chains. Antisera were also prepared to the purified protein of the first case of alpha chain disease (T. L.), to γG and γM, and to several κ- and λ-Bence Jones proteins. All were made specific by suitable absorption. Antisera to κ and λ chains were selected which precipitated both with Bence Jones proteins and γA or γG-myeloma proteins of the corresponding light-chain type. A monkey antiserum specific for human γA2-proteins (13) was kindly provided by Dr. H. G. Kunkel. Antisera to human colostral γA were kindly provided by Doctors T. B. Tomasi and J. P. Vaerman. When absorbed with concentrated normal human serum, one of these antisera reacted only with the "secretory piece," whereas the other reacted also with lactoferrin.

Immunoelectrophoresis was performed with 2% agar gel in barbital buffer, ionic strength = 0.05, pH 8.2. Ouchterlony agar diffusion studies were carried out in 1.5% agar in 0.15 M NaCl at pH 7.2. Quantitative immunoglobulin determinations were performed by the radial diffusion technique (14) by use of standard calibration curves. The inhibition of precipitin reactions was detected by a screening test in gel diffusion as previously described (15).

γ-globulin fractions from the specific antisera were prepared for *immunofluorescent studies* by standard techniques (16). Several anti-γA sera including one against T. L. protein and a number of anti–light-chain sera were used, whereas only one anti-γG and anti-γM sera were conjugated. The specificity of the absorbed antisera was carefully tested

before conjugation, as stated above. The specificity of the fluorescein-conjugated globulins was tested on stored bone marrow smears (known to contain numerous plasma or lymphoid cells) from patients with myeloma or macroglobulinemia of well established class and type. Several peroral suction-biopsy jejunal fragments were obtained from the four patients. Rectal biopsy specimens and surgical small intestine biopsies were available from two patients. The specimens were usually quick-frozen and sectioned (4 μ) in a cryostat. The sections were air-dried, fixed for 5 min in absolute methanol, and promptly stained. Some specimens were previously fixed in neutral isotonic buffered formaldehyde (10%) for 4 hr at 4°C, followed by a 30% sucrose solution wash overnight, according to Eidelman and Davis (17). A few specimens were stored at −80°C for later processing. Bone marrow was studied in all patients. White cells were washed three times in a 5% bovine albumin solution in Hanks' buffer. The smears were fixed in ethanol for 3 min and immediately stained. Mesenteric lymph nodes were available from two patients. One of them was necrotic and not usable. The lymph node cells, obtained by gentle teasing, were washed and processed exactly as bone marrow cells. Sections and smears were incubated with the labeled antisera by standard methods (16). A rhodamine-conjugated normal rabbit fraction II was used as a counterstain.

In vitro protein synthesis by the same tissues was studied by the technique of Hochwald, Thorbecke, and Asofsky (18). The specimens of small intestine, rectum, and mesenteric node biopsies, as well as the bone marrow cells, were submitted to short-term (24–36 hr) culture in the presence of ¹⁴C-labeled amino acids (1 μc/ml of a mixture of L-valine-¹⁴C, L-leucine-¹⁴C, and L-phenylalanine-¹⁴C). The concentrated cell extracts were analyzed by immunoelectrophoresis and subsequent radioautography. Normal serum and the patient's serum were usually used as carriers; some experiments were performed without any carrier.

Isolation of the pathological protein and physicochemical studies

Preparative procedures. Preparative zone electrophoresis was performed in Pevikon C 870[2] under an electric potential of 7 v/cm⁻¹ for 48 hr at +4°C, using barbital acetate buffer, pH 8.6, and ionic strength 0.05 with 20% v/v glycerol to insure good quality of imprints for protein-staining as previously described (19).

Gel filtration chromatography was performed on a 100 cm × 3.5 cm I.D. column, packed with Sephadex G200 equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, 1 mole/liter in NaCl, and 1% in N-butanol. To eliminate traces of anthrone-positive material, the Sephadex had been repeatedly boiled and washed in distilled water. The ratio of the applied sample volume to the total Sephadex bed volume was kept near 1:100.

In view of the considerable electrophoretic heterogeneity and tendency towards polymerization of the alpha chain disease proteins, a three step schedule was adopted for its purification: (a) the broad electrophoretic band containing the "alpha chain protein" (α-CP) was eluted after preparative electrophoresis in Pevikon blocks; (b) after removing the low density lipoproteins by ultracentrifugation, this block fraction was submitted to Sephadex G200 gel filtration. The first major and asymmetrical peak eluting between Ve/Vo 1 and 1.4 contained mainly the α-CP polymers together with α2M and small amounts of γM; (c) the 1st two-thirds of

this peak were collected and fractionated again by Pevikon block electrophoresis. The segments (1 cm wide) were eluted, concentrated, and tested for α2M and γM contaminants. Some of these fractions were found to contain only the α-CP when tested in gel diffusion at a concentration of 20 mg/ml. The final yield of purified material was less than 5% of the starting material.

Mild reduction of the polymeric forms of the α-CP was performed at room temperature either with 0.3 M 2-mercaptoethanol for 1 hr in 0.5 M Tris-HCl buffer, pH 8.0, or with 0.01 M dithiothreitol for 30 min in 0.2 M Tris-HCl buffer, pH 8.6. This was followed by alkylation with a 10% equivalent excess of iodoacetamide for an equal period of time at 0°C. The reduced and alkylated proteins were dialyzed in acetylated 23/32 Visking bags against distilled water for 4 hr and then again against the desired solvent overnight in the cold (except for some experiments in 6 M guanidine HCl, 1 mole/liter in acetic acid where the dialysis time was 48 hr at room temperature). In some experiments, α-CP were extensively reduced by 0.05 M dithiothreitol in 8 M urea rendered 0.5 mole/liter in Tris-HCl buffer, pH 9.0. The reduction was stopped after 1 hr at room temperature with a 10% equivalent excess of iodoacetamide for 30 min. After dialysis for 24 hr against several changes of 1 M acetic acid, the material was lyophilized.

Human γA-myeloma proteins were isolated by block electrophoresis followed by G200 Sephadex gel filtration in phosphate-buffered saline, pH 7.2. In some instances, the γA was further purified by chromatography on carboxymethylcellulose, using a gradient of acetate buffer at pH 5.0 from 0.29 to 0.074 mole/liter. Light and heavy chains were prepared following the procedure of Fleischman, Pain, and Porter (20), using a Sephadex G100 column equilibrated in 1 M acetic acid (or in some experiments in 3 M guanidine HCl) to separate the reduced and alkylated chains. Although only the mid-third of the first peak was selected for the heavy-chain preparations, only some of these preparations, especially those from λ-chain proteins, were essentially pure when tested immunologically.

Analytical procedures. Concentrations of the isolated α-CP or their subunits were measured by absorption at 280 mμ using an extinction coefficient $E_{1\,cm}^{1\%} = 9.4$. This value was established for the protein T. L. by determination of its dry weight and was arbitrarily used for the other α-CP preparations. Total proteinuria in these patients was measured by the biuret reaction after precipitation by trichloracetic acid at a final concentration of 20% at boiling point. For protein-bound carbohydrate determinations, total hexoses were measured by anthrone reaction according to Mokrasch (21), D-mannose and D-galactose in 1:1 molar ratio being used as standard. Fucose was assayed according to the method of Dische and Shettles (22), using L-fucose as standard. Sialic acid was determined with thiobarbituric acid reagent according to Warren (23), using N-acetylneuraminic acid as standard. Total hexosamines were determined using the Elson-Morgan reaction as described by Boas (24); D-glucosamine converted as free base was used as standard. To evaluate the role of sialic acid moieties on its electrophoretic heterogeneity, mildy reduced and alkylated T. L. protein was treated with neuraminidase (Behring)[3] incubating 1 U/μg of sialic acid for 15 min at 37°C; the reaction was stopped by freeze-drying.

Analytical ultracentrifugation was carried out with a Spinco model E. ultracentrifuge at 59,780 rpm, at 20°C. Sedimentation coefficients were calculated by standard meth-
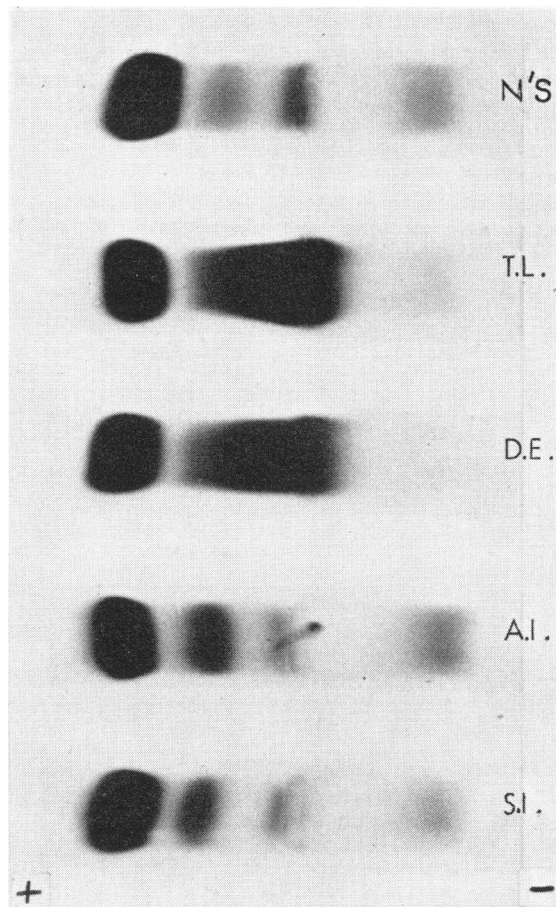

FIGURE 1 Agar-gel electrophoretic patterns of the serum of the four patients compared with normal serum (N[1] S).

ods. Density gradient ultracentrifugation with 10–40% sucrose gradients was performed according to methods outlined before (25). Agar-gel electrophoresis was effected in 1% agar in barbital buffer, pH 8.2, ionic strength 0.05, and the slides were stained with amido black. Vertical starch[4]-gel electrophoresis was performed either in borate buffer, pH 8.6, according to Smithies (26) or in 0.05 M formic acid, 0.01 M sodium hydroxide, pH 3.5, 8 moles/liter in urea, according to Poulik (27).

To study the polymeric forms of α-CP and to determine the molecular weight of the monomers, gel filtration experiments were performed on Sephadex G100 and G200 equilibrated with an aqueous solution of 6 M guanidine HCl, 1 mole/liter in acetic acid by upwards elution in columns 80 cm × 2.5 cm I.D. The optical densities of the solvents at 280 mμ were less than 0.03. The Sephadex G200 column was calibrated by measuring the Ve/Vo values of the following proteins: human myeloma γG, human γ-polypeptide chains, hog gastric pepsin, trypsin, human κ-polypeptide chains, and horse heart cytochrome c. The Ve/Vo values were plotted vs. log mol wt.

Attempts to recombine heavy and light chains of γA-myeloma proteins and to combine light chains with the

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monomer of the α-CP T. L. were performed according to Grey and Mannik (28). The reduced and alkylated chains (or monomers) were first dialyzed separately against 0.1 M acetic acid. The mixtures were prepared in this solvent in a ratio of H (or monomer) : L = 2 : 1 on the basis of protein concentration. Subsequently they were dialyzed against distilled water, followed by 0.01 M Tris-HCl buffer, pH 8.0. Each step of dialysis was carried out for 20 hr at 4°C using a 100-fold excess of the solvent. The total protein concentration of the mixtures during the dialysis steps was approximately 1.0 mg/ml.

RESULTS

The serum electrophoretic pattern of patients T. L. and D. E. was strikingly abnormal, showing a broad band which extended from α2- to β2-globulins, a decrease in serum albumin possibly due to severe intestinal malabsorption, and a profound hypogammaglobulinemia (Fig. 1). In both patients the concentration of this fraction was approximately 4 g/100 ml at the time of first study. Immunoelectrophoretic analysis with polyvalent

antisera to normal human serum revealed an abnormal precipitin line (Fig. 2 A) extending from the α1-globulins to the slow β2-region, crossing at its extremities the serum albumin and γG lines. This abnormal protein reacted with all antisera to γA (Fig. 2 B).

In the other two patients, no abnormal band was detectable in the serum electrophoretic pattern (Fig. 1). There was only an increase of the α2-globulins, contrasting with a decrease of all other protein fractions, and in most samples a moderate hypogammaglobulinemia. The γA abnormality could have escaped routine immunoelectrophoretic analysis with antiserum to whole normal serum, especially in case S. I. (Fig. 3). However, immunoelectrophoretic analysis with antisera to γA showed an abnormally fast precipitin line (Fig. 3 B), which was hidden amongst the α2-globulin lines and barely visible when using the polyvalent antisera (Fig. 3 A).

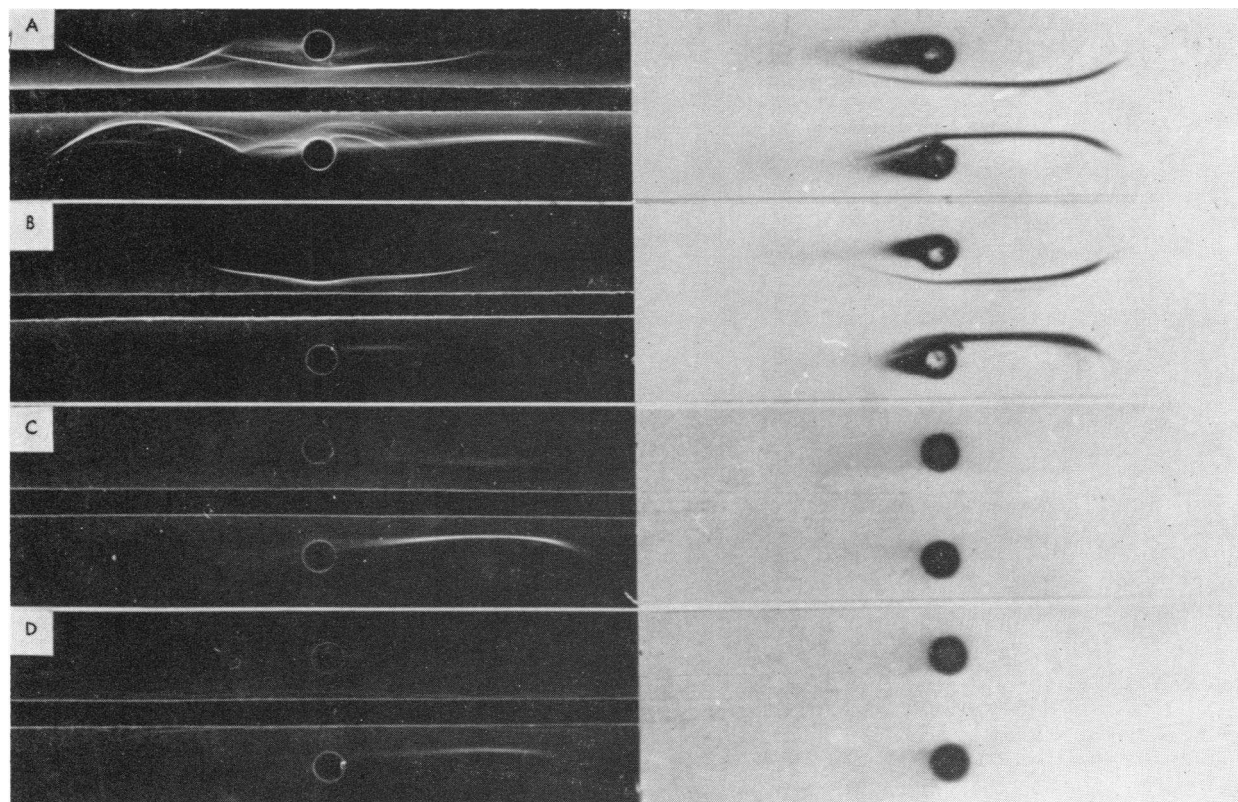Table I indicates the γG and γM levels in successive serum samples of the four patients. In all cases, κ- and

FIGURE 2   (Left) Immunoelectrophoresis of 0.5 μl of serum of patient D. E. (top wells) compared with 1 μl of normal serum (lower wells) with A: antiserum to whole normal serum; B: antiserum to γA; C: antiserum to κ-light chains; and D: antiserum to λ-light chains. The anode is to the left. (Right) Radioautographic patterns of an immunoelectrophoretic analysis of a cell extract from an intestine biopsy culture of patient D. E. in presence of ¹⁴C-labeled amino acids. The sera, used as carriers, and the antisera are the same that appear at the left. Note that the only labeled line is that corresponding to γA- and α-chain disease protein (α-CP) and that there is no reaction with the antisera to light chains.

λ-light chains were present in γG and γM in a roughly normal ratio. Immunochemical quantitation of the pathological γA-protein by radial diffusion could not give reliable figures because of its tendency to polymerize (see below) and because of the lack of valid standard for a calibration curve.

Antigenic analysis of the serum protein. In each of the four patients, the anomalous γA component was not seen in immunoelectrophoretic patterns developed with several antisera specific for κ- or λ-light chains (Fig. 2 C and D). Moreover, the isolated pathological protein did not combine with anti-κ or anti-λ antibodies and did not inhibit their precipitin reaction with Bence Jones proteins. Immunoelectro-
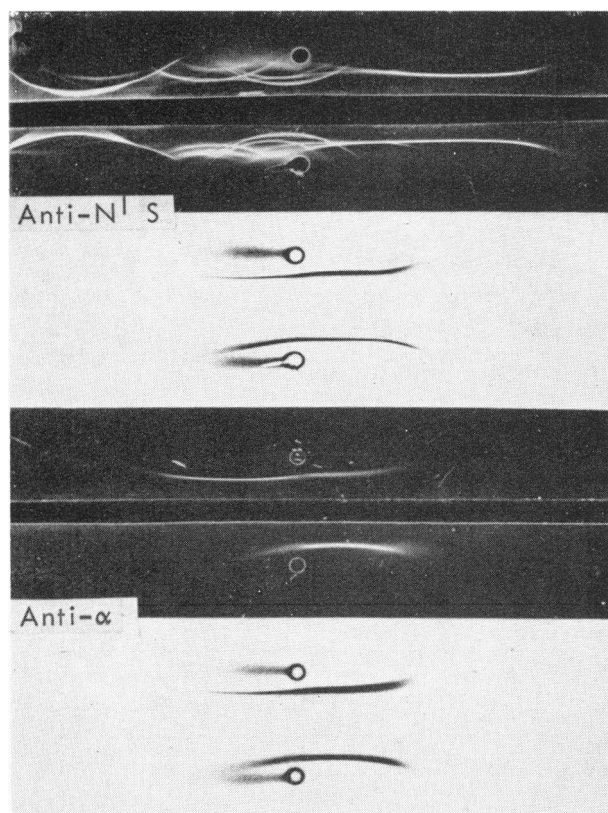

FIGURE 3 Immunoelectrophoretic analysis and subsequent radioautography of a cell extract from an intestinal biopsy culture of patient S. I. in presence of ¹⁴C-labeled amino acids. The carriers were 1 μl of serum of the patient S. I. (top wells) and 1 μl of normal human serum (lower wells). Note: (*a*) that the fast α-CP precipitin line in serum S. I. is easily detected with antiserum to γA (anti-α) but escapes to analysis with antiserum to whole normal serum (anti–N¹S) because it is hidden among the α2-globulin lines; (*b*) that the only labeled line is that corresponding to γA and to α-CP; (*c*) that the slight spur of normal γA over α-CP in serum S. I. (middle pattern with anti-γA) is not seen on the radioautograph. In this particular experiment, the anodic end of the α-CP precipitin line is not labeled.

TABLE I
γG and γM Serum Levels

Patient	Date	γG	γM
		mg/ml	*mg/ml*
T. L.	Oct. '67	6	0.82
	Apr. '68	4.3	0.70
D. E.	Aug. '68	3.3	0.66
	Jan. '69	6.1	0.84
	Apr. '69	6.7	0.63
S. I.	Mar. '68	10.3	0.98
	Dec. '68	6.1	0.68
	Apr. '69	9.6	0.63
A. I.	June '68	7.2	0.66
	Oct. '68	9.6	0.70
	Apr. '69	10.6	1.20

The normal serum values in this laboratory are: 11.7 mg/ml ±2.7 for γG and 1.2 mg/ml ±0.5 for γM.

phoretic analysis revealed slight binding of albumin in only one subject to the abnormal protein and no binding of haptoglobin in any of the four. With some of the antisera monospecific for γA, a faint line was detected inside the abnormal protein line in sera T. L. and D. E. (Fig. 4). This second precipitin line was shown to correspond to normal γA-globulins. For sera A. I. and S. I., the normal γA-line spurred over the fast abnormal line when the same antisera were used (Fig. 4). Double diffusion experiments and cross-absorption studies with the same antisera confirmed that, in all four cases, the isolated pathological protein was antigenically deficient when compared with normal γA. The antigenic properties of the four abnormal proteins were compared in Ouchterlony analysis with those of several γA-myeloma proteins of known light-chain types. All antisera were strictly specific for γA and had been absorbed with light chains. Depending upon the antiserum, four patterns were found (Fig. 5): (*a*) with most antisera, the α-CP were in complete identity with all myeloma globulins (Fig. 5 C); (*b*) with some antisera, γA-myeloma proteins with κ-chains, but not those with λ-chains, spurred over the α-CP, although the precipitin lines of the myeloma proteins of both light-chain types fused completely (Fig. 5 A); (*c*) inversely, other antisera showed a spur of γA-myeloma proteins of type L, and not of those of type K over the pathological proteins (Fig. 5 B); (*d*) when using two of our antisera made against K γAl-myeloma protein, α-CP were found to be deficient when compared with all tested γA-myeloma proteins of type K as well as of type L (Fig. 5 D). However, even this latter pattern was due to antibodies related to conformational specificity and light-heavy chain interaction since the same antisera showed complete identity between
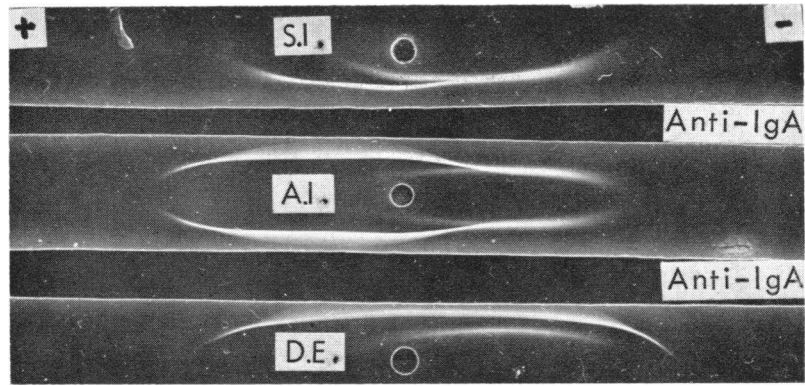
FIGURE 4 Immunoelectrophoresis of sera S. I., A. I., and D. E. using a specific antiserum to γA which contains antibodies reacting with the light-heavy chain combination. These patterns show in all sera a precipitin line corresponding to normal γA. This line spurs over the α-CP line of sera S. I. and A. I. at their cathodic extremity, whereas it is inside the α-CP line for serum D. E.

the α-CP and the purified heavy chains from the myeloma proteins. Moreover, after absorption of these antisera with the purified α-CP, no more reaction was detectable with the purified myeloma heavy chains, whereas these absorbed antisera gave a precipitin reaction with the γA-myeloma proteins. Thus, the antigenic deficiency of α-CP vs. γA-myeloma proteins demonstrated in these experiments, although possible related to Fd reactivity, is mainly due to the absence of light chains.

None of the γA-myeloma proteins studied in our laboratory have yielded Fc-fragments after papain hydrolysis. We were therefore unable to compare directly the α-CP to these fragments. No antigenic determinants common to the pathological proteins and Fab fragments of γA-myeloma proteins could be demonstrated. Attempts to digest the purified α-CP polymers by papain in standard conditions in the presence of 0.01 M cystein hydrochloride under standard conditions were ineffective. When performed in the presence of 0.1 M 2-mercaptoethanol, papain degradation resulted in the production of small peptides without any immunologically identifiable component.

The pathological proteins of all four patients were shown to belong to the α1-subclass (Fig. 6). When antisera demonstrating the antigenic deficiency of γA2-myeloma proteins were used, the α-CP gave a reaction
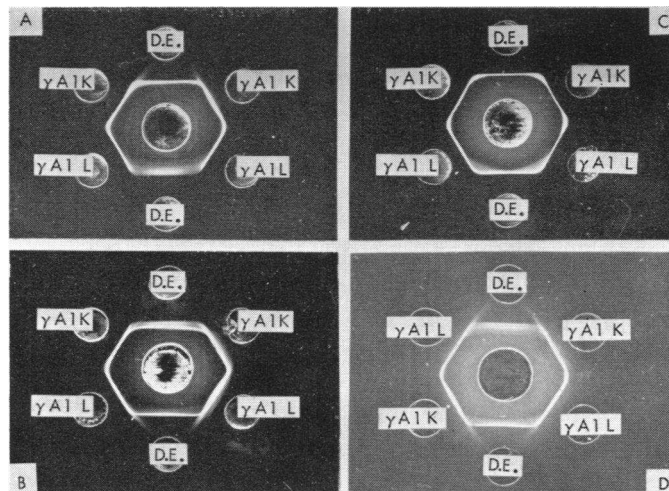

FIGURE 5 Ouchterlony plates showing antigenic comparison between α-CP of patient D. E. and four γA1-myeloma globulins with known light-chain type. The central wells contain four different antisera to γA. Antisera used in A and B were selected because they contained potent antibodies to α-κ and α-λ combination, respectively. The four patterns are described in the text.

of identity with γA1-myeloma proteins and spurred over γA2-myeloma proteins (Fig. 6 A). None of the purified α-CP (up to a concentration of 10 mg/ml) precipitated with an antiserum specific for the γA2-subclass (Fig. 6 B). Immunoelectrophoretic experiments showed that, in contrast, the normal γA-globulin of patient S. I. contained a small proportion of γA2-molecules.

Antisera to T. L. protein did not demonstrate any individual antigenic specificity of this protein and failed to precipitate with Fab-fragments of γA-myeloma proteins. The antigenic properties of each of the four α-CP were compared by double diffusion analysis and cross-absorption experiments, using 15 different antisera to γA1-myeloma proteins and four antisera to T. L. pro-
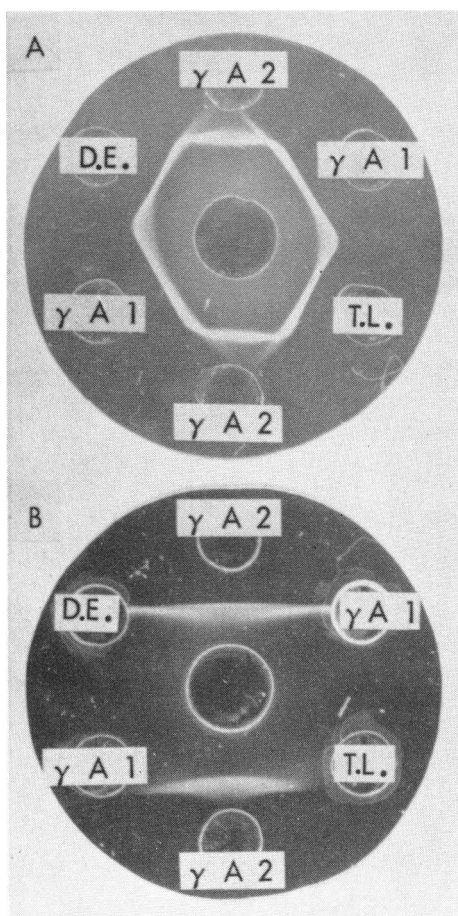

FIGURE 7 Ouchterlony test useful for identification of α-CP. The selected antiserum to γA in the center well is that used in Fig. 5 D; it shows that the protein under study (X) is an α-CP, since it is deficient when compared to normal γA- and γA1-myeloma proteins of both light-chain types, while in identity with the reference protein T. L.

tein. No antigenic differences among the four proteins were detected by these antisera.

In view of the results of this antigenic analysis, a simple Ouchterlony analysis test, illustrated in Fig. 7, was devised and has proven useful for the diagnosis of alpha chain disease when this possibility is suggested by immunoelectrophoretic analysis with antiserum to γA, especially if only a small amount of serum is available. In this test, the isolated protein or the diluted serum is compared with γA1-myeloma proteins of both light-chain types, normal γA and a reference α-CP, using an absorbed antiserum to γA containing antibodies which precipitate only when κ- or λ-chains are combined with α-chains.

Physicochemical studies. Fig. 8 A shows a typical elution chromatogram obtained when an alpha chain disease serum was submitted to Sephadex G200 filtration in Tris buffer. A large proportion of protein eluted in the first peak between Ve/Vo 1 and 1.4. The same elution pattern was obtained whether or not serum had been collected in iodoacetamide and whether or not it had been stored. While the 1st peak contained the bulk of the α-CP, α-CP was also found in appreciable amounts in the 2nd peak, together with γG and other proteins. The 3rd peak was devoid of the pathological protein except for serum T. L. where it was present in moderate amounts. Ultracentrifugal analysis of these sera confirmed that the pathological protein was polydisperse with a series of peaks with sedimentation coefficients ranging from 4S to 11S. The pattern was identical when serum had been collected in iodoacetamide. However, in contrast to the chromatographic data, the analytical ultracentrifugal pattern contained a major "4S" peak (57% for serum T. L. and 80% for serum D. E.), and density

FIGURE 6 Subclass typing of proteins T. L. and D. E. by comparison with γA1- and γA2-myeloma proteins. The antiserum to γA used in the central well of the upper plate (A) was selected because it showed antigenic deficiency of the γA2-proteins, whereas it contained no antibodies specific for the light-heavy chain combination. In the lower plate (B), the antiserum was specific for γA2-proteins and did not react with T. L. and D. E. proteins at a concentration of 10 mg/ml.
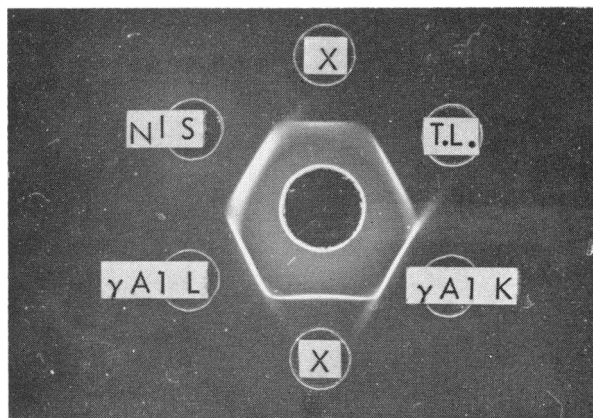
gradient ultracentrifugation showed that the α-CP was widely distributed along the gradient and was present in fractions heavier than γG as well as in those containing albumin.

When the block electrophoresis fraction containing the αCP was subjected to Sephadex G200 gel filtration, a large fraction of the pathological protein was eluted in the first asymmetrical peak (shown on Fig. 8 B), together with γM- and α2M-contaminants. Since the small amount of normal γA present in the serum was eluted in the last third of this 1st peak (fraction 3 of the figure), only the first two-thirds of the peak were pooled for further purification. Purification was achieved for all the α-CP except protein S. I. Ultracentrifugal analysis of this purified material showed considerable mass heterogeneity with several peaks ranging from 8S to 12S. When submitted to urea-acid–starch-gel electro-

FIGURE 9 Vertical starch-gel electrophoresis in 8 M urea formiate buffer, pH 3.5 of: 1, α-CP of patient A. I.; 2, the same reduced and alkylated; 3, isolated heavy polypeptide chains of a γA1-myeloma protein; 4, isolated K light chains of the same γA1-myeloma protein; 5, another γA1-myeloma protein reduced and alkylated; 6, α-CP of patient D. E. reduced and alkylated; and 7, unreduced α-CP of patient D. E. In all cases reduction was performed with 0.01 M dithiothreitol.
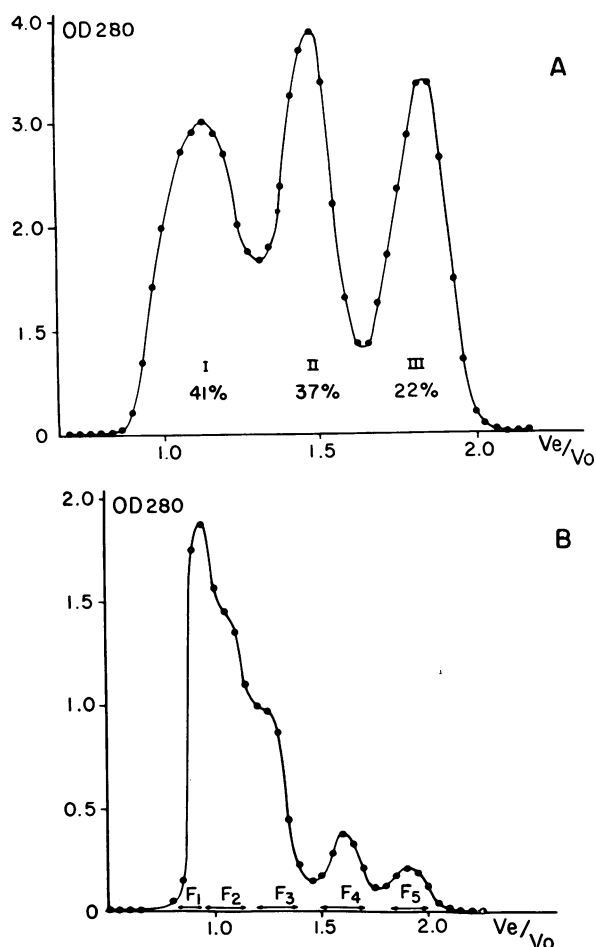

FIGURE 8. Elution patterns from gel filtration chromatography in Sephadex G200 columns equilibrated with 0.1 M Tris-HCl buffer, pH 8.0, 1 mole/liter in NaCl. (A) Serum D. E. (B) Pevikon block electrophoretic fraction of serum T. L. containing the α-CP.
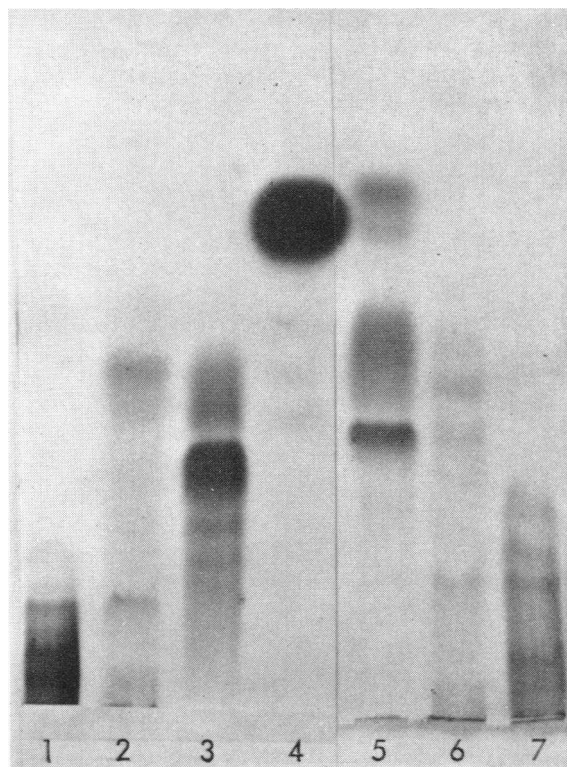
phoresis, this polymerized material penetrated into the gel poorly (Fig. 9). When run on a Sephadex G100 column in 6 M guanidine, more than 50% of this material was recovered in the void volume. After mild reduction and alkylation, the purified protein still displayed electrophoretic heterogeneity in agar and gave multiple bands in acrylamide-gel electrophoresis. In a few experiments, ultracentrifugal analysis of this reduced and alkylated protein showed a single peak with a sedimentation coefficient of 3.2S–3.3S. However, in other experiments, the ultracentrifugal pattern showed polydispersity. The strong tendency of this mildly reduced material to re-aggregate was also demonstrated when it was submitted to filtration on Sephadex G100 or Bio-Gel P150 [5] columns. Since most of the material eluted in the void volume, it was not possible to free the pathological protein from γM subunits and α2M contaminants by this method. When this reduced and alkylated material was

[5] Bio-Rad Laboratories, Richmond, Calif.

TABLE II

Carbohydrate Content of Proteins T. L., D. E., and A. I. and of the α-Heavy Chain of a γA1-Myeloma Globulin

	Total hexoses	Fucose	Sialic acid	Total hexosamines
		mg/100 mg protein		
Protein T. L.	6.67	0.84	3.72	5.36
D. E.	6.64	0.38	6.00	7.60
A. I.	4.56	<0.20	2.60	3.66
α-chain of γA1-myeloma	3.19	0.53	0.79	2.80

submitted to Sephadex G200 filtration in 6 M guanidine HCl, 1 mole/liter in acetic acid, 80% of the material was eluted with a Ve/Vo = 1.8. These results indicate that a characteristic feature of the α-CP is its great tendency to polymerize and that it is present in native serum in multiple polymeric forms of a basic monomeric structure held together both by disulfide bonds and strong non-covalent forces.

When the mildly reduced and alkylated purified α-CP from patients T. L., D. E., and A. I. were submitted to urea-acid-starch-gel electrophoresis, the characteristic band of the light polypeptide chains was always lacking and the "heavy-chain band" was diffuse (Fig. 9). Extensively reduced and alkylated T. L. protein gave the same electrophoretic pattern in urea-acid starch gel.

Gel filtration experiments with the reduced and alkylated α-CP on a calibrated Sephadex G200 column in 6 M guanidine HCl, 1 M acetic acid allowed a rough estimate of the molecular weights of the monomeric units of the purified proteins. The values obtained were approximately 36,000, 38,000, and 38,000 for T. L., A. I., and D. E., respectively.

Attempts to combine the monomeric units of T. L.- and D. E.- α-CP with purified light chains of γA-myeloma proteins were unsuccessful, whereas the control experiments performed with the same light chains and alpha chains of heterologous myeloma proteins yielded recombined molecules.

The carbohydrate content of the three purified α-CP, as compared to α-chains of a γA1-myeloma is indicated in Table II. The strikingly high concentration of sialic acid and hexosamines in proteins T. L. and D. E. (confirmed when studying the urinary protein) was not found for protein A. I.

T. L. protein monomer was digested with neuraminidase and then submitted to agar-gel electrophoresis and to starch-gel electrophoresis in borate buffer, pH 8.6. Although the anodic mobility was considerably reduced as compared with that of the untreated material, there was no significant reduction in the degree of charge
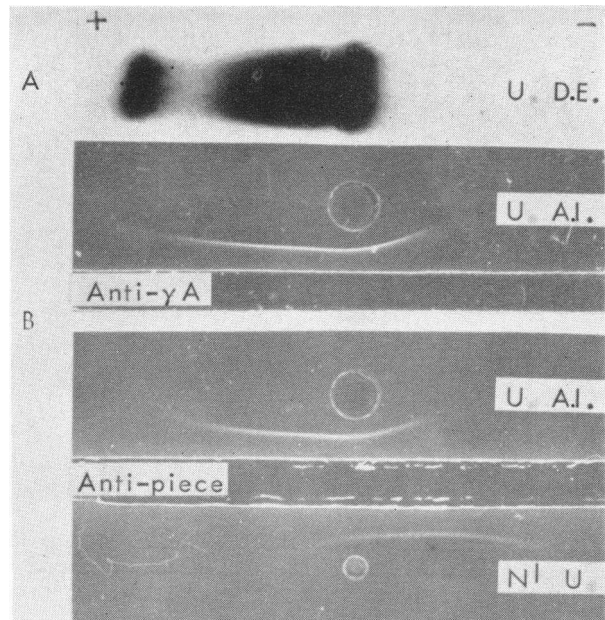

FIGURE 10 Urinary α-CP analysis. (Top) Agar-gel electrophoresis of the concentrated urinary proteins of patient D. E. (U D. E.). (Below) Comparative immunoelectrophoretic analysis of concentrated urinary proteins of patient A. I. (U A. I.) with antiserum to γA and antiserum to secretory piece (Anti-piece). The lowest well contains normal concentrated urine (N¹U) showing the secretory γA-line.

heterogeneity after neuraminidase treatment which had removed all sialic acid moieties.

Study of the urine, jejunal fluid, and saliva protein. Since α-CP are poorly precipitable in sulfosalycilic acid and 10% trichloracetic acid, probably owing to their high carbohydrate content, quantitative estimates of the proteinuria by these routine methods were inaccurate. The thermosolubility test for Bence Jones proteins was always negative. The amount of protein excreted in the urine was generally low and varied from day-to-day. It ranged between 0.05 and 1 mg/ml for patient T. L. and between 0.15 and 0.50 mg/ml for patient D. E., except for one sample which contained 1.8 mg/ml. In patients S. I. and A. I., total proteinuria never exceeded 0.2 mg/ml. In all four cases, the α-CP was found in concentrated urines. For cases T. L. and D. E., the electrophoretic pattern showed a broad abnormal band (Fig. 10 A) whose electrophoretic mobility and heterogeneity were similar to that of the serum protein, small amounts of albumin, and other proteins. The antigenic and physicochemical characteristics of this urinary protein were identical with those of the pathological serum protein. The agar electrophoretic pattern of concentrated urines of patients A. I. and S. I. was unremarkable. However, immunoelectrophoretic analysis showed the abnormal precipitin line with antisera to γA. The urinary com-

ponent of these two patients was somewhat less heterogeneous and less anodic than the serum component. This urinary protein was shown to be devoid of light chains and antigenically identical with the serum α-CP. In all four cases, only trace amounts of free light chains of both antigenic types were detected in the urine. The urinary α-CP of patients A. I. and S. I. was shown to be bound to the secretory piece, as illustrated in Fig. 10 B, whereas the α-CP in serum did not react with antisera to secretory piece.

Jejunal fluid was studied for all patients except T. L. The α-C.P. was always detected and immunoelectrophoretic analysis showed that, in all the studied samples, this protein was the main component reacting with antisera to whole normal human serum. The precipitin arc revealed by antisera specific for γA was similar to that of the serum pattern. Here again, the pathological protein was shown to lack light chains and to be associated with the secretory piece.

The abnormal protein was never found in parotid saliva in the several specimens obtained from patients D. E., S. I., and A. I. For patient T. L., the α-CP was shown to be present in significant amounts (4), but only total saliva had been studied. In this case, however, studies of total saliva are inconclusive as illustrated by the fact that patient D. E. exhibited α-CP in total saliva although not detected in its parotid saliva. This may simply reflect an inflammatory state of the buccal mucosa. Normal secretory γA was shown to be present in decreased amounts in all parotid saliva samples except for the initial sample of one of the patients.

In the course of these studies, we found that the antibodies reacting specifically with the α-light-chain combination were unable to precipitate with normal secretory γA in saliva, urine, or intestinal fluid. However secretory γA does inhibit the precipitin reaction of these antibodies with serum γA. This finding suggests that the conformational specificity of the Fab region is modified when secretory piece is bound to the γA-molecule (29).

Cellular synthesis studies. The results of the immunofluorescence study of small intestine biopsies were essentially similar for all four patients (Fig. 11). When the sections were treated with conjugated antisera to γA, a diffuse or patchy fluorescence caused by extracellular material was seen in many specimens (Fig. 11 A). The cytoplasm of the proliferating lymphoid and plasma cells forming a dense infiltrate in the lamina propria was either faintly fluorescent (Fig. 11 B) or negative (Fig. 11 C). On a surgical biopsy specimen of one of the patients, it was clear that the fluorescent cells were not evenly distributed, but localized in some zones. Very occasional brightly fluorescent cells stained by anti-γA antiserum were seen, in contrast to the poorly stained

sheets of proliferating cells. We vainly attempted to increase the brilliance of this cytoplasmic fluorescence by using different technical variations including conjugated anti-T. L. protein antiserum and the indirect method with unconjugated antisera to γA. Results were constantly negative both for the extracellular material and for the proliferating cells with several anti–light-chain antisera, apart from an exceptional bright cell clearly seen on the dark background and presumably synthesizing a normal Ig molecule. γG-producing cells were practically absent, while γM cells were somewhat more frequent, sometimes in small clumps in the vicinity of eosinophils.

Radioimmunoelectrophoretic analysis of the proteins synthesized in vitro by the same small intestine biopsy specimens demonstrated the production of the α-CP in all instances (Fig. 2 and 3). Multiple biopsy specimens for each of the four patients were studied by this technique and the γA precipitin line was always strongly labeled when normal serum was used as carrier. When the serum of the corresponding patient was used as carrier, the α-CP line was also strongly labeled. In several instances, the use of a carrier serum was unnecessary because the amount of labeled α-CP present in the cellular extract was sufficient to give a precipitin line which was shown to be antigenically deficient when compared with normal γA. For most specimens, γA was the only labeled immunoglobulin line when normal serum was used as carrier. Occasionally γG and/or γM lines were faintly labeled. The α-CP synthesized in vitro never reacted with antisera to κ- or λ-light chains in contrast to normal secretory γA in control experiments performed with normal intestine. No free labeled light chains could be demonstrated in the cell extracts, even if light chains were used as carriers. A rectal biopsy of patient A. I. showed involvement by the proliferative process and the corresponding specimens also synthesized the α-CP in vitro.[6]

Mesenteric lymph nodes removed at surgery from patients A. I. and S. I. were studied by the same technique. In both cases, the synthesis of a significant amount of α-CP was demonstrated and the use of a carrier serum for the radioimmunoelectrophoretic analysis was unnecessary. When normal serum was used as carrier, γA

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[6] In the course of these experiments, we found that all the small intestinal or rectal specimens from two patients synthesized in uncontaminated cultures a component with α1-mobility. Although strongly labeled on the radioautographs, the corresponding line was not detectable on the stained plates. This component was seen whether or not human serum was used as carrier. It was revealed by most but not all rabbit antisera, by pooled normal rabbit serum, pooled normal human serum, purified normal γG, but not by human agammaglobulinemic sera. The hypothesis of a blood group substance reacting with rabbit and human γG was considered but has not been substantiated by preliminary experiments.
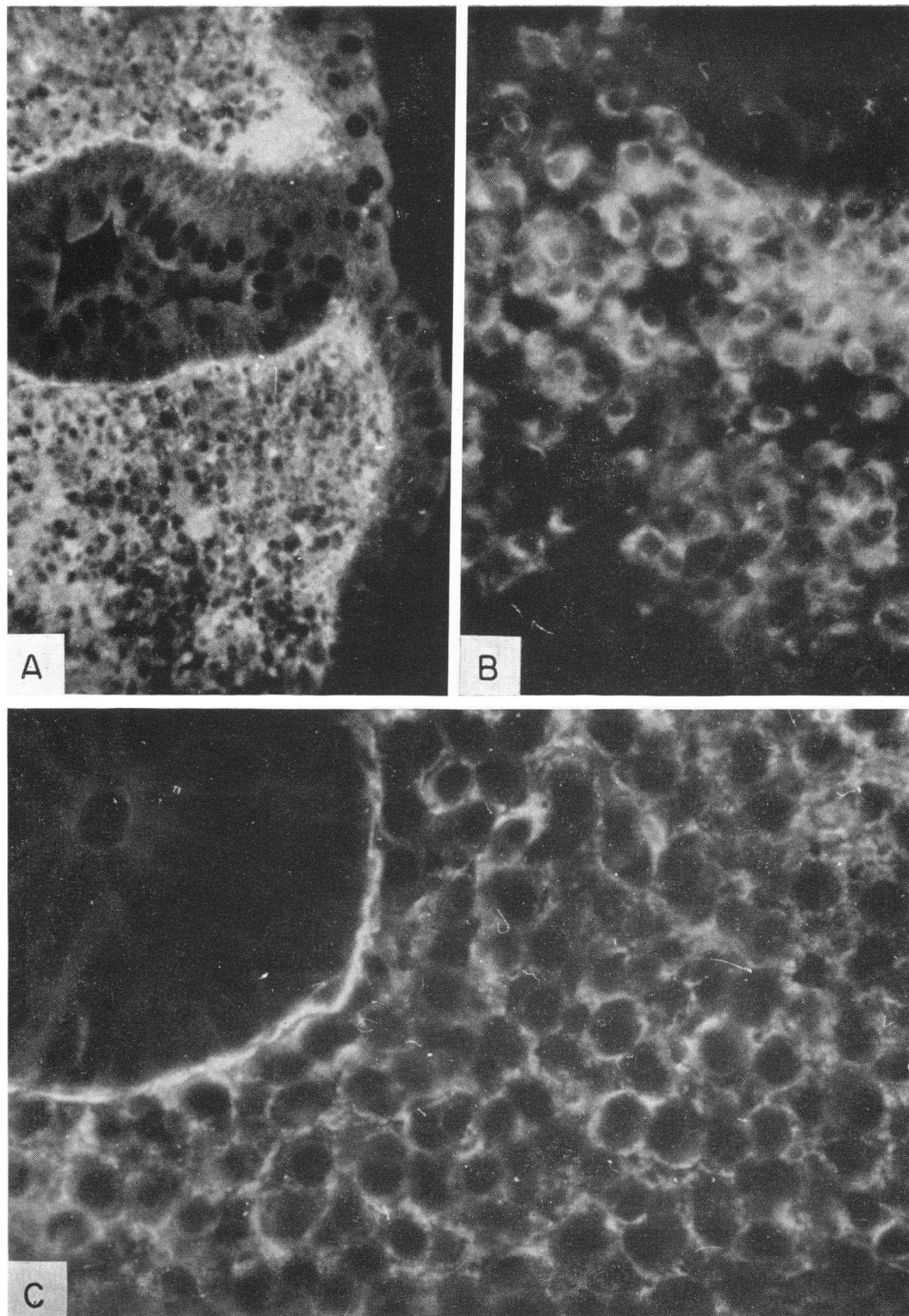
FIGURE 11 Jejunum cryostat sections of patient D. E., stained with fluorescein-labeled anti-α antiserum. (A) Diffuse and mainly extracellular fluorescence, (B) weak cytoplasmic fluorescence, and (C) peri or extracellular fluorescence, the cytoplasm of the plasma cells being negative.
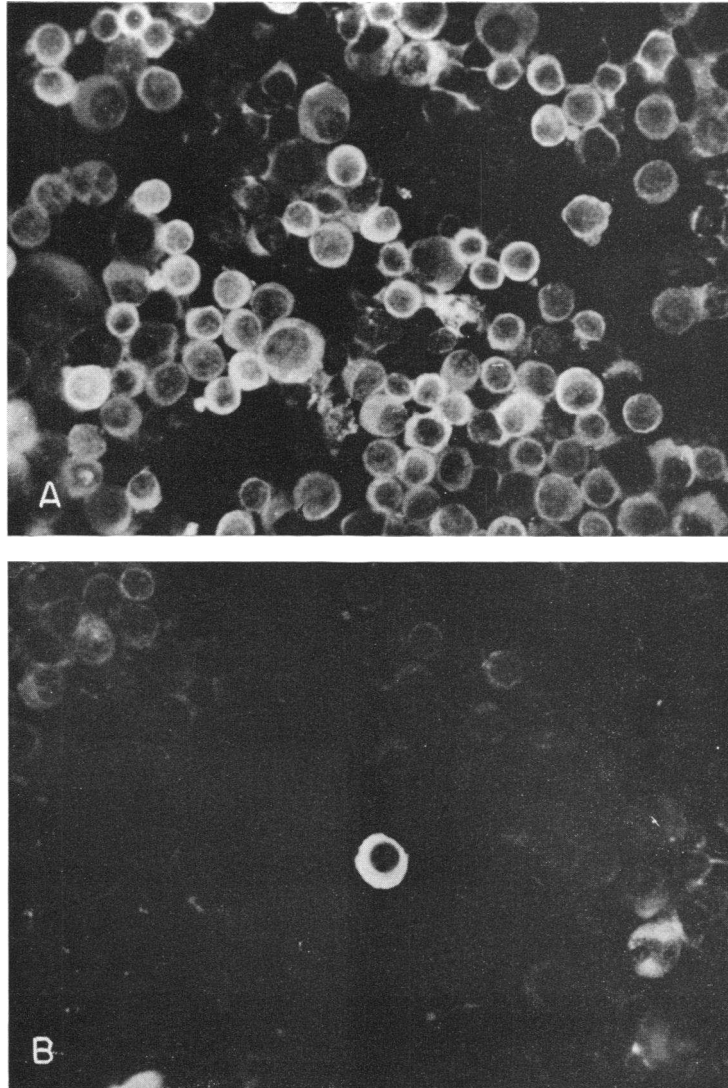
FIGURE 12 Lymph node cells smears of patient A. I., stained: (A) with anti-α–conjugated antiserum, more than half of the cells are positive and show varying degrees of brightness and (B) with anti-K–conjugated antiserum, a single plasma cell brightly fluorescent.

was the only immunoglobulin line labeled by the cell extract of patient S. I., whereas γG was faintly labeled in the experiment with the node of patient A. I. The immunofluorescence study of the lymph node cells of patient A. I. is illustrated in Fig. 12. With the antisera to γA, approximately half of the cells were positive. The stained cells were either plasma cells or medium to large lymphoid cells. Although the result was more clear-cut than with intestine sections, the cytoplasmic fluorescence was not very intense and one could notice cells with varying degrees of brightness (Fig. 12 A). With anti-

sera to κ- and λ-light chains, the cells were negative, apart from one exceptional bright cell presumably synthesizing a normal complete immunoglobulin (Fig. 12 B).

Bone marrow smears showed a normal immunofluorescence pattern for all patients except T. L., and no in vitro synthesis of α-CP was detected by raidoimmunoelectrophoretic analysis. The immunofluorescence study of the bone marrow of patient T. L. demonstrated a moderate involvement by the neoplastic process. On three occasions, 4–6% of the nucleated cells (half plasma

cells and half medium to large lymphoid cells) were positive with the antisera to γA, whereas the reactions were consistently negative with anti-κ and anti-λ antisera, except for an extremely small number of cells corresponding to γG- and γM-producing cells. In vitro synthesis experiments confirmed the production of a small amount of T. L. α-CP.

DISCUSSION

The present studies show that the anomalous protein of alpha chain disease is in many respects similar to that of γ-heavy-chain disease (2, 30–33). The lack of light chains was demonstrated by several immunological and chemical methods. It should be emphasized that the absence of precipitation with antisera to light chains is not a sufficient criterion since such a failure to precipitate has been encountered in this and other (34) laboratories with several γA-myeloma proteins, mainly with λ-chains. Rabbit antisera containing antibodies which give precipitin reactions only when light and α-heavy chains are combined were found to be especially useful for detection of the pathological protein without purification. In addition to antisera with antibodies specific for the α-κ or α-λ combination (35), we found during this study that some antisera contained antibodies reacting with the combination of α-chains with any κ- or λ-light chains. These findings confirm the importance of the light-heavy chain interaction in establishing the antigenic structure of the immunoglobulin molecules (36, 37).

The molecular weight estimations in calibrated Sephadex column indicate that the three studied abnormal proteins represent only a portion (roughly two-thirds) of α1-chain. These data have been recently confirmed by precise molecular weight determinations by the meniscus depletion method of Yphantis in guanidine.[7] That α-CP are related to the Fc-fragment of γA is indicated by antigenic analysis and by the presence of a carboxy-terminus similar to that of myeloma α-chains.[8] The lack of at least a portion of the Fd-piece could not be convincingly demonstrated by immunological techniques with the available antisera because of the poor immunogenicity of the Fd piece. However, the inability of the anomalous proteins to combine with light chains suggested that Fd piece or a portion thereof is missing. Further structural studies are required in order to determine precisely the length and location of the segment of Fd piece which is missing in these α-CP. The study of the NH2-terminal region is of the utmost importance since diverging data have been found for γ-heavy-chain

[7] Dorrington, K. J., and M. Seligmann. In preparation.
[8] Mihaesco, Edith. Unpublished data.

disease proteins. Prahl (38) found in one of these proteins an N-terminal sequence identical to that of complete γ-heavy chains, suggesting that chain synthesis is normally initiated and that there is a gene deletion. In contrast, the data recently reported by Ein, Buell, and Fahey (33) for two other γ-heavy-chain disease proteins indicated that the variable part of the N-terminal portion of the normal γ-chain is missing.

The large amount of carbohydrate in T. L. and D. E. proteins recalls the original observation for the γ-heavy-chain disease protein CR (9), and remains to be explained. The striking electrophoretic heterogeneity of the α-CP is presumably not due to the sialic acid moieties, as demonstrated by Grey for one of the γ-chain disease proteins (39). This electrophoretic heterogeneity may be partly related to the very high tendency of α-CP to polymerize. The polymers held together by noncovalent bonds in addition to disulfide bonds were shown to occur in vivo. The discrepancy between the ultracentrifugal and gel filtration data remains to be explained. The tendency towards polymerization may explain the low output of the anomalous protein in urine.

When studied with antisera to γA, the antigenic properties of the anomalous protein found in urine and, in significant amount, in jejunal fluid of all patients were similar to that of the serum protein. The finding that the α-CP in urine and jejunal fluid of two patients was associated with secretory piece suggests that piece is bound to the Fc-region of the secretory γA-molecules.

All four proteins belonged to the α1-subclass which constitutes about 85% of the normal γA-molecules (13). Although these four α-CP are immunologically indistinguishable with the available antisera, structural differences between them may be detected by future chemical studies. That these proteins were "monoclonal" in nature, despite their electrophoretic heterogeneity, could be proven only by the negative reactions with the antiserum specific for the γA2-subclass. This monoclonal character is in good agreement with the neoplastic nature of the cellular infiltration in the gut and the involved lymph nodes (4, 7–9).

The cellular studies clearly show that the α-CP is synthesized by the proliferating cells present in the small intestine (all patients), mesenteric nodes (2/2 patients), bone marrow (1/4), and rectum (1/2). Electron microscopic studies for patients D. E. and A. I. substantiated that most of these cells were plasma cells (8, 9). In addition, the present studies have confirmed in all four patients that there is no detectable light-chain synthesis at the intracellular level (3). A similar observation has been recently made in a patient with γ-heavy-chain disease (33). However, in this latter study the radio-immunoelectrophoretic experiments were performed with

the supernatant fluid of the cultures, whereas in the present work the cell extracts were used and were shown to be devoid of free labeled light chains. The data indicate that this is not an absence of light-heavy chain assembly and suggest that the genes coding for light chains (and possibly part of the heavy chain) are not expressed in these tumor cells. If confirmed by studies of nascent immunoglobulin subunits on polysomes, these findings would imply that the incomplete heavy chains are released from the polysomes and secreted despite the absence of light chains. This is in contrast to the current hypothesis on the mechanisms of intracellular assembly (40, 41) and secretion (42) of immunoglobulin molecules. While the findings in these patients do not invalidate these hypotheses, they suggest that at least in certain abnormal situations light chains are not required to ensure release of heavy chains and transport across the rough endoplasmic reticulum. The faint positivity of the proliferating cells found in all four patients by immunofluorescent study with antisera to γA remains to be explained. A possible explanation for this unexpected finding is that the abnormal alpha chain might be rapidly secreted from the cells, so that the net intracellular pool of this protein would as a result, be low. The low periodic acid-Schiff (PAS) positivity of the majority of the tumor cells (4) contrasting to the high carbohydrate content of α-CP is in agreement with this hypothesis. In view of recent reports on the possible role of carbohydrate in the Ig secretion (43), the postulated fast release of α-CP from the cells could be related to its very high carbohydrate content. In addition, the sugar moieties could possibly protect the Fc-region of a nascent intact α-chain from an intracellular proteolytic process, thus leading to the presence of α-CP. Further biosynthesis studies are required to clarify these points.

That alpha chain disease primarily involves the intestinal tract is not an unexpected finding in view of the importance of the digestive lymphoid tissue in the γA-synthesis (44). However, it is in contrast to the rarity of gastrointestinal involvement in disseminated myeloma (45) and, in our experience, in γA-myelomas. The clinicopathological features (4, 7–9) were strikingly similar in the four patients herein studied and similar to those in the so-called "Mediterranean" type of abdominal lymphoma described by Israeli authors (5, 6, 46) who emphasized that, in their experience, this type of lymphoma occurred only in young Arabs and non-Ashkenazi Jews. Two of our patients with α-chain disease were Kabyles from Algeria, one a Syrian Arab, and the fourth a Eurasian with a French father and a Cambodian mother. A fifth patient with α-chain disease, also found in a Paris hospital and very recently diagnosed in this laboratory, is also a young Algerian. This striking predi-

lection for some populations may be a result of the action of environmental factors, such as intestinal microorganisms, or of a genetic predisposition similar to that found in Waldenström macroglobulinemia (47) or of both. A study of immunoglobulins of the relatives of our patients with α-chain disease has been undertaken and the first results are uninformative. Two other important questions remain: (a) Will α-CP abnormality be found in clinical syndromes other than lymphoma of the digestive tract? (b) Do most or only few cases of "Mediterranean" type of abdominal lymphoma represent α-chain disease? Unfortunately, none of the previously described cases included an immunoglobulin analysis. However, a case recently reported by Jinich, Rojas, Webb, and Kelsey (48) in an Irakian Jew living in Mexico City could have been an unrecognized α-chain disease since "hypergammaglobulinemia" of 3.6 g/100 ml was recorded. Through the courtesy of Doctors Bracha Ramot (Tel-Aviv), Zlotnick (Jerusalem), and Hobbs (London), we have been able to identify α-CP in the serum of four additional patients with this type of abdominal lymphoma. Thus, it seems highly probable that, although previously unrecognized, α-chain disease is less infrequent than γ-heavy-chain disease.[*]

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[*] Several other cases of α-chain disease have been recognized in Algiers since this manuscript was submitted.

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