

Isolation of a Novel Glycoprotein from the Urine of a Patient with Chronic Myelocytic Leukemia

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ABSTRACT Patient B. J. with chronic myelocytic leukemia excreted 0.5–1.1 g protein per day in the urine. Gel filtration on Sephadex G-75 showed about one-third of this protein to be in molecular weight range 20,000–40,000 (fraction BJC). BJC, prepared from 9 liters of urine by gel filtration, was chromatographed on carboxymethylcellulose. Two proteins were eluted from the resin in pure form (as shown by zone and immunoelectrophoresis) in yields representing 8 and 3 mg/liter of urine: BJC1 and BJC2. Their amino acid compositions were identical. BJC1 contained 61% carbohydrate (33% hexose, 11% sialic acid, 13% glucosamine, 5% galactosamine). BJC2 contained one-fourth to one-half as much of each carbohydrate. Molecular weight of BJC1 was estimated at 29,000 by gel filtration. Neither glycoprotein reacted with rabbit antiserum to normal human serum.

Antiserum to BJC1 was made in the rabbit. Immunoelectrophoresis with this antiserum showed a faint precipitin line, corresponding in mobility to BJC1, in normal human plasma, and a stronger line in most leukemic plasmas. By immunodiffusion, BJC1 was not detectable in normal human urine, but a positive reaction occurred in the following conditions: leukemia, 64–72%; other types of disseminated neoplastic disease, 36–78%; regional ileitis, 45%; ulcerative colitis, 38%; tuberculosis, 33%; during the 1st wk after major surgery, 33%.

BJC2 was found in the urine by immunoelectrophoresis in 10% of patients with neoplastic disease and was not observed in urine of other patients or in human plasma.

Amino acid composition, carbohydrate content, and antigenic specificity indicate BJC1 is a previously unrecognized member of the system of normal human

plasma glycoproteins. Like certain other glycoproteins, its plasma concentration frequently increases in patients with neoplastic disease, chronic inflammatory disease, or tuberculosis and after surgery. Because molecular weight is 29,000, increased plasma concentration readily causes its appearance in the urine.

INTRODUCTION

A previous study here showed that patients with advanced neoplastic disease frequently excrete in the urine proteins in molecular weight range 10,000–50,000 which do not react with antiserum to normal human serum (1). 25 such proteins were visualized in urine from nine patients with disseminated cancer (1) but were not isolated or characterized.

The present study focused on one patient with chronic myelocytic leukemia in whom "low molecular weight proteinuria" was present. A homogeneous glycoprotein not previously described was isolated, its chemical composition was determined, a specific antiserum was produced, and with this antiserum the frequency of urinary excretion of the novel glycoprotein was studied in the general clinical population.

METHODS

The subject, patient B. J., was a 66-yr-old Negro female with a 6-mo history of anorexia, weight loss, and dyspnea on exertion. Clinical evaluation revealed chronic myelocytic leukemia and hypertensive heart disease with congestive failure. Urine protein was reported "2+." On day 11, treatment was begun with busulfan and blood transfusions. Staphylococcal bronchopneumonia developed on day 12, and despite penicillin treatment the patient died on day 15. Chronic myelocytic leukemia was confirmed at autopsy.

To purify and characterize the urinary proteins, these methods were used: (a) gel filtration on 2 × 200- and 4 × 200-cm columns of Sephadex G-75 (2); (b) ion-exchange chromatography on 2 × 30-cm columns of carboxy-

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TABLE I
Incidence of Positive Immunodiffusion Reactions of Urine with Antiserum to BJC1 in Various Groups of Patients*

Group	Diagnosis	No. of cases	Positive reaction %
I	Normal	38	0
II	Acute myelocytic leukemia	14	64
III	Acute lymphocytic leukemia	16	75
IV	Chronic myelocytic leukemia	12	67
V	Chronic lymphatic leukemia	14	72
VI	Hodgkin's disease (stages III and IV)	18	78
VII	Carcinoma of the ovary (metastatic)	12	50
VIII	Carcinoma of the pancreas (metastatic)	13	54
IX	Carcinoma of the breast (metastatic)	20	40
X	Carcinoma of the lung (metastatic)	18	39
XI	Malignant melanoma (metastatic)	14	36
XII	Alcoholic cirrhosis	22	4
XIII	Congestive heart failure	18	6
XIV	Essential hypertension	12	8
XV	Peptic ulcer	11	9
XVIA	Herniated nucleus pulposus (preoperative)	12	0
XVIB*	Herniated nucleus pulposus (2-5 days postoperative)*	12	33
XVII	Regional enteritis	9	45
XVIII	Ulcerative colitis	8	38
XIX	Active pulmonary tuberculosis	6	33
XX	Growth hormone deficiency	11	0
XXI	Muscular dystrophy	14	0
XXIIA	Uterine fibromyoma (preoperative)	10	0
XXIIB*	Uterine fibromyoma (2-8 days postoperative)*	10	33

* Same patients as corresponding group A

methylcellulose (CM)¹ 11 (3); (c) acrylamide-gel electrophoresis at pH 8.9 and 4.0 (4); (d) paper electrophoresis at pH 9.0 (5); (e) immunodiffusion (at pH 7.2) (6); and (f) immunoelectrophoresis (at pH 8.6) (7) against these antisera (Hyland Div., Travenol Laboratories, Inc., Costa Mesa, Calif. and Behringwerke Laboratories, Somerville, N. J.): rabbit antisera to whole human serum, and rabbit, horse, or goat antisera to 16 purified human plasma proteins, these being α -lipoprotein, transferrin, α_2 -haptoglobin, fibrinogen, albumin, γ G-immunoglobulin, β_1 C/ β_1 A-globulin, α_1 -antitrypsin, α_2 -HS-glycoprotein, hemopexin, β_2 C-glycoprotein, α_1 acid glycoprotein, β -lipoprotein, Gc globulins, α_2 -macroglobulin, and C-reactive protein; (g) measurement of molecular weight by gel filtration on a calibrated Sephadex G-75 column (2); (h) analysis of amino acid composition (8); (i) analysis of content of hexoses (9), glucosamine (after hydrolysis with 2 N HCl at 100°C for 6 h under vacuum [10]), and sialic acid (11).

Antisera to proteins isolated from the urine of B. J. were prepared by injecting the protein, suspended in Freund's adjuvant, into rabbits according to route, dose, and schedule of Campbell, Garvey, Cremer, and Susdorf (12).

¹ *Abbreviations used in this paper:* CEA, carcinoembryonic antigen; CM, carboxymethylcellulose; WBC, white blood cells.

These antisera were applied in double immunodiffusion to random urine samples from 38 normal volunteers (age 6-70 yr) and 284 patients on medical, surgical, and pediatric services at Emory University and Henrietta Egleston Hospitals (see Table I for diagnoses). These individuals were arranged in 22 groups. Within each group, cases were numbered consecutively (e.g., in group I, 1 through 38). Subjects were identified by group and by case number within group, e.g., case I/6 is sixth individual in group I.

Antisera prepared to urinary proteins of B. J. were also tested in double immunodiffusion against nine purified human plasma proteins (Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.): albumin, immunoglobulin G, fibrinogen, transferrin, α_1 -acid glycoprotein, ceruloplasmin, prealbumin, haptoglobin, and β_2 C glycoprotein.

Antiserum to isolated urinary protein was incorporated into immunoplates according to Mancini, Carbonara, and Heremans (13). With these immunoplates, concentration of the antigen in plasma and in phenol extracts of white blood cells (WBC) was determined by single radial immunodiffusion (13).

WBC were collected in 50-300 mg batches from normal individuals by a continuous-flow cell separator. Similar quantity of WBC was obtained from leukemic subjects by allowing 10-30 ml anticoagulated blood to settle for 30

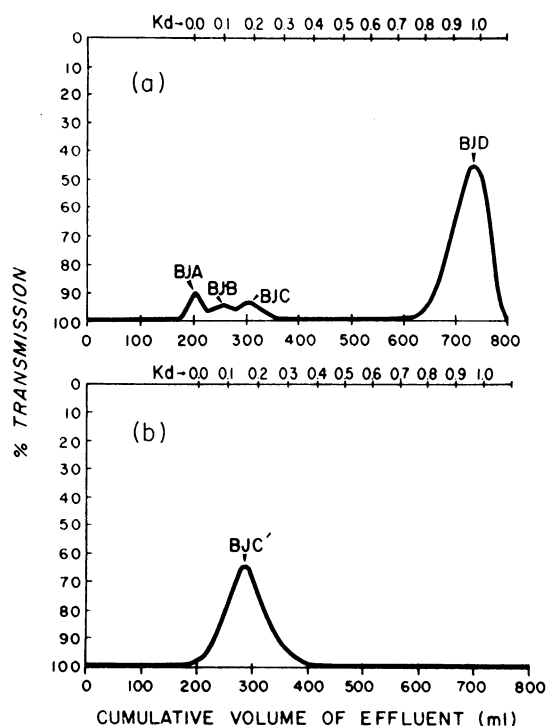


FIGURE 1 Elution profiles from chromatography of urine preparations on 2×200 cm column of Sephadex G-75. Vertical axis shows percent transmission of light at 280 nm. (a) 5 ml urine of patient B. J.; (b) 20 mg fraction BJC'.

min and removing the WBC layer.² WBC were washed three times with 0.9% NaCl and then extracted with phenol according to Springer, Nagai, and Tegtmeyer (14): the phenol extract was dialyzed against H_2O and lyophilized, yielding 6–9 mg/100 mg WBC (wet weight) in both leukemic and normal cells. This material was dissolved in 0.9% NaCl at a concentration of 1–10 mg/ml, and was tested against appropriate antiserum to urinary protein in double immunodiffusion and in single radial immunodiffusion.

RESULTS

Urinary proteins were isolated from patient B. J. during days 2 through 10 of hospitalization. During this period, the patient excreted 0.5–1.1 g urine protein per day. By cellulose acetate electrophoresis, the composition of this protein was 36% albumin, 15% α_1 -globulin, 20% α_2 -globulin, 16% β -globulin, and 13% γ -globulin. Analysis of 5 ml urine by gel filtration on Sephadex G-75 (Fig. 1a) showed four peaks: "BJA," eluted with distribution coefficient (K_d)³ 0–0.03, cor-

² WBC preparations were made by Dr. W. R. Vogler, Department of Medicine, Emory University School of Medicine.

³ $K_d = (V_e - V_o)/V_t$, where V_e is elution volume of test substance, V_o is void volume, and V_t is volume of solvent imbibed by the gel.

responding to mol wt $> 69,000$ (2); "BJB," $K_d = 0.03$ –0.13, approximate mol wt 40,000–60,000; "BJC," $K_d = 0.13$ –0.27, mol wt 20,000–40,000; and "BJD," $K_d = 1.0$, representing UV-absorbing substances with mol wt $< 1,000$. Normal human serum shows only peaks "A" and "D"; normal human urine contains only peak "D"; urine from patients with intrinsic renal disease shows the same pattern as normal serum (1).

Attention was now focused on peak BJC. For preparative purposes, the urine was lyophilized in batches of 300 ml. The resulting powder was dissolved in 50 ml 1 N acetic acid, chromatographed on a 4×200 -cm column of Sephadex G-75, and BJC recovered by lyophilization. This material was purified three times by gel filtration on the 2×200 cm column, after which it emerged as a single symmetric peak with median $K_d = 0.15$ (fraction "BJC'," Fig. 1b).

Acrylamide-gel electrophoresis showed four visible components in BJC'. This preparation, however, did not react with antiserum to human serum in immunodiffusion, or to any of the 16 specific antisera to purified human plasma proteins.⁴ An antiserum to BJC' was produced in the rabbit. With this antiserum, immunoelectrophoresis revealed at least four components in BJC' (Fig. 2a).

BJC' was now analyzed by ion-exchange chromatography on CM-11 (Fig. 3), with elution of five fractions (BJC1 \rightarrow BJC5). Each was recovered by lyophilization and examined by acrylamide-gel electrophoresis and immunodiffusion. BJC1 and BJC2 were homogeneous by electrophoresis both in acrylamide gel and on paper (Fig. 4); the other fractions contained two or more components. No fraction reacted with antiserum to whole human serum, or with specific antisera to human plasma proteins. Immunoelectrophoresis

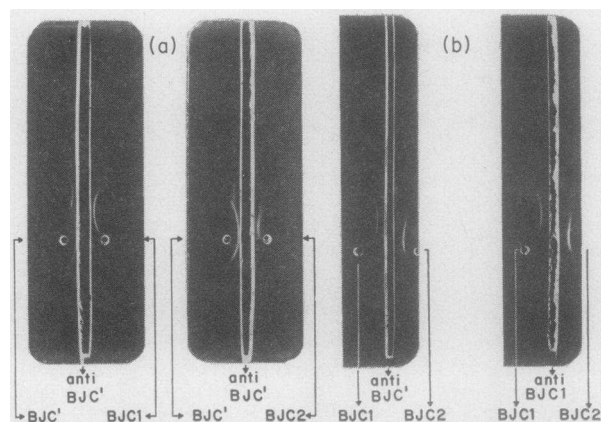


FIGURE 2 Immunoelectropherograms of BJC', BJC1, and BJC2.

⁴ The 16 specific antisera are identified in Methods.

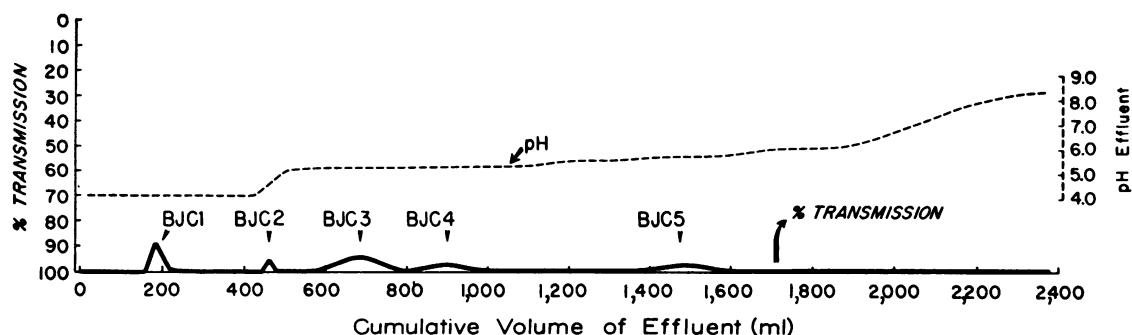


FIGURE 3 Elution profile from chromatography of 169 mg fraction BJC' on 2×30 cm column of CM-11. Mode of elution was as described in reference 3, with addition that at cumulative volume 1,900 ml, eluting solvent was changed to pH 8.0, 1.0 M ammonium acetate.

versus antiserum to BJC' confirmed homogeneity of BJC1 and BJC2 (Fig. 2a). Each of the other three fractions showed two or more precipitin lines.

Attention was now directed to BJC1 and BJC2. The yield of BJC1 averaged 8 mg/liter of urine. 9.3 liters of urine were available and 75 mg BJC1 was obtained. The yield of BJC2 was only 26 mg. Remaining BJC fractions are still under purification.

Studies on BJC1. Criteria of homogeneity were: (a) BJC1 moved as a single component in acrylamide gel at pH 8.9 and at pH 4.0 (Fig. 4); (b) BJC1 moved as a single component in paper electrophoresis at pH 9.0; the band stained with the fuchsin reagent for glycoprotein as well as with bromphenol blue reagent for protein; and (c) BJC1 reacted with antiserum to fraction BJC' as a single component in immunodiffusion and immunoelectrophoresis (Figs. 2a and 2b). It did not react with any of three rabbit antisera to normal

human plasma or with specific antisera to 16 plasma proteins. 1 mg BJC1 contained 0.26 ng carcinoembryonic antigen according to radioimmunoassay (15).⁵

Amino acid and carbohydrate composition of BJC1 are given in Table II. Carbohydrate constituted 61% of the glycoprotein. Molecular weight was estimated by gel-filtration to be 29,000.

Periodic injection into rabbits of BJC1 in Freund's adjuvant produced an antiserum to the glycoprotein. This serum reacted with BJC1 as a single component in immunoelectrophoresis and immunodiffusion (Fig.

TABLE II
Amino Acid and Carbohydrate Composition of Proteins
BJC1 and BJC2

Amino acid	BJC1	BJC2
Lysine	44 \pm 2.9	50 \pm 2.0
Histidine	11 \pm 0.5	13 \pm 0.6
Arginine	33 \pm 1.1	39 \pm 2.7
Aspartic acid	110 \pm 4.0	106 \pm 2.2
Threonine	68 \pm 2.5	64 \pm 2.2
Serine	81 \pm 3.0	78 \pm 1.0
Glutamic acid	128 \pm 3.2	138 \pm 2.9
Proline	87 \pm 3.0	81 \pm 3.1
Glycine	114 \pm 4.7	117 \pm 4.0
Alanine	72 \pm 3.8	65 \pm 2.4
$\frac{1}{2}$ -Cystine	60 \pm 2.1	65 \pm 3.4
Valine	53 \pm 2.3	48 \pm 2.3
Methionine	12 \pm 1.0	17 \pm 1.0
Isoleucine	18 \pm 1.1	22 \pm 1.3
Leucine	55 \pm 2.5	54 \pm 2.4
Tyrosine	23 \pm 1.5	20 \pm 0.7
Phenylalanine	30 \pm 1.1	24 \pm 1.6
Hexose	33.3 \pm 2.3	14.6 \pm 1.0*
Sialic acid, %	10.5 \pm 0.5	3.7 \pm 0.3*
Glucosamine, %	12.8 \pm 0.9	3.1 \pm 0.2*
Galactosamine, %	4.8 \pm 0.3	1.2 \pm 0.2*

Amino acid contents are expressed as residues per 1,000 residues, excluding tryptophan which was not measured. Values represent average \pm SE for analyses of three preparations of each protein

* $P < 0.05$ for comparison between BJC1 and BJC2.

⁵ This analysis was performed by Dr. Jack Snyder, Hoffmann-LaRoche Laboratories, Nutley, N. J.

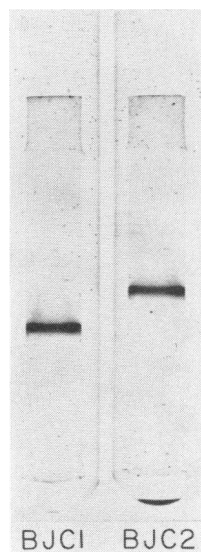


FIGURE 4 Electrophoresis at pH 8.9 in acrylamide gel. The gels were stained with Amido Schwarz.

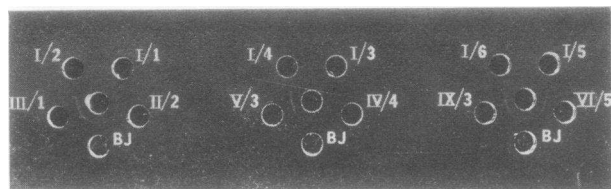


FIGURE 5 Immunodiffusion of urine samples versus anti-BJC1. Samples are designated thus: I/2 is urine of subject 2, group I (Table I).

2b). Further studies with this antiserum are described below.

Studies on protein BJC2 were limited by the quantity available. Acrylamide-gel electrophoresis at pH 8.9 showed one component with mobility slower than that of BJC1 (Fig. 4). Immunoelectrophoresis against antiserum to either BJC' or to BJC1 showed a single precipitin arc cathodal to that of BJC1 (Figs. 2a and b). In amino acid composition BJC2 was indistinguishable from BJC1, but it contained only 25-45% as much hexose, sialic acid, and hexosamine (Table II). 15 mg of BJC2 in Freund's adjuvant was injected in divided doses at 2-wk intervals in one rabbit, but precipitating antibody did not develop.

Observations with antiserum to BJC1. Immunoelectrophoresis versus normal plasma showed a faint arc with the same mobility as BJC1. This line was more intense in the majority of about 20 leukemic sera tested (acute myelocytic, acute lymphocytic, chronic

myelocytic, chronic lymphocytic). An arc corresponding to BJC2 was not visible. The antiserum did not react in double immunodiffusion with nine available human plasma proteins.^a

In immunoelectrophoresis, B. J.'s urine showed two arcs corresponding to BJC1 and BJC2; her serum showed only one arc corresponding to BJC1.

Double immunodiffusion was also carried out with antiserum to BJC1 versus urines from 38 healthy and 284 diseased individuals (Table I, Fig. 5). (a) No normal urine reacted. (b) 64-72% of urines from leukemic patients were positive. (c) Urine from 36-78% of patients with six other types of disseminated neoplastic disease reacted. (d) Positive reactions were observed in 33-45% of patients with chronic inflammatory disease (tuberculosis, regional ileitis, ulcerative colitis) and during the 1st wk after two types of surgery.

Analysis of plasmas by single radial immunodiffusion against antiserum to BJC1 are illustrated in Fig. 6. The results showed the following. (a) For both BJC1 and BJC2, an approximately linear relationship between (diameter)² of the precipitate area and concentration of glycoprotein was obtained. However, the slope of the line and its intercept on vertical axis were lower for BJC2 than for BJC1 (Fig. 6). (b) For normal and leukemic plasmas, the concentration of

^a Identified in Methods.

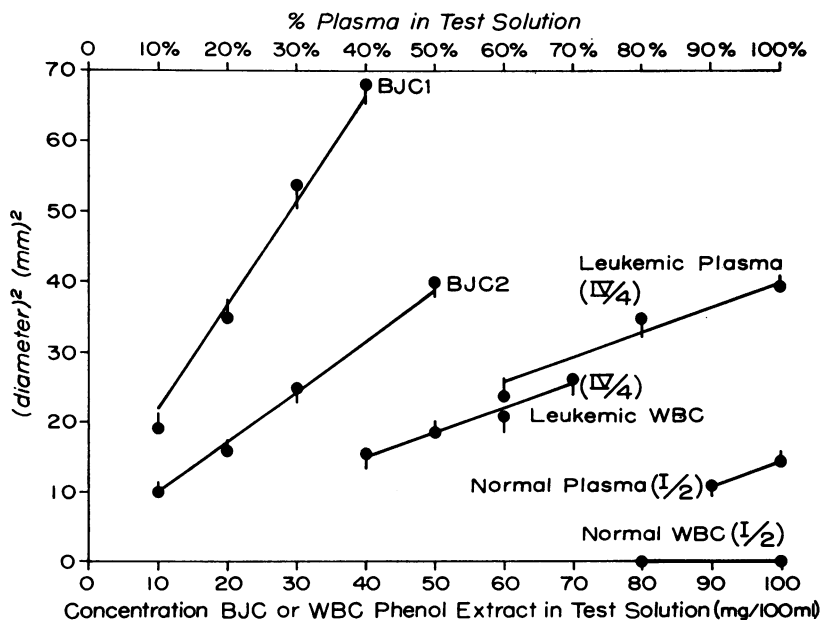


FIGURE 6 Single radial immunodiffusion with antiserum to BJC1. Each point shows average \pm SE of three measurements. Plasmas were diluted to specified extent with 0.9% NaCl before analysis. Plasma and WBC samples are identified as in Fig. 5.

BJC1 in plasma (average \pm SE) was 12.4 ± 1.2 ($n = 7$) and 30.3 ± 3.1 ($n = 10$) mg/100 ml, respectively.⁷

Phenol extracts of WBC from four normal individuals did not react in double or single radial immunodiffusion with antiserum to BJC1. Extracts from four of six patients with chronic myelocytic leukemia reacted in both tests. The precipitin line in double diffusion showed a reaction of identity with both BJC1 and BJC2. Only BJC1 was visible in immunoelectrophoresis. By single radial immunodiffusion, concentrations of BJC1 in the four leukemic WBC preparations which contained immunoreactive material were estimated in the range 0.5–1.8 mg/100 mg WBC (wet weight)⁸ (Fig. 6).

DISCUSSION

Leukemic patients frequently excrete low molecular weight proteins in the urine (1). Of the two classes (BJB and BJC) of low molecular weight urinary protein in BJ, one (BJC) was fractionated and found to contain five or more proteins different⁹ from the 33 presently recognized proteins (16) of human plasma. Of these five urinary proteins, two were isolated: BJC1 and BJC2. The former is a glycoprotein which can be distinguished from the known plasma glycoproteins¹⁰ on the basis of (a) uniquely high carbohydrate content (61%), (b) distinctive amino acid composition, and (c) antigenic specificity. At first we thought BJC1 is not present in normal human plasma because the glycoprotein does not react in double immunodiffusion or immunoelectrophoresis with rabbit antiserum to human plasma. This conclusion, however, was reversed when the specific BJC1 antiserum showed a faint line of reaction in immunoelectrophoresis with

normal human plasma. A stronger line of reaction with most leukemic plasmas indicated that the plasma concentration of BJC1 is generally increased in leukemic patients. This conclusion was confirmed by single radial immunodiffusion (Fig. 6). Frequent (64–75%) presence of BJC1 in leukemic urine presumably results from the elevated plasma concentration exceeding the renal threshold.

BJC1 is not detectable by double immunodiffusion in normal urine, but a substance with its antigenic and electrophoretic properties occurs in urine of 33–75% of patients with disseminated neoplastic disease, chronic inflammatory disease of the gastrointestinal tract, and tuberculosis. BJC1, or a closely similar substance, also frequently appears in the urine during the first week after two types of major surgery.

Normal human plasma contains at least 20 glycoproteins. The total level of protein-bound carbohydrate, and the concentration of one glycoprotein easily measurable (α_1 -acid glycoprotein), rise in patients with neoplastic disease, and after surgery and other types of trauma (17, 18). The present experiments add a previously unrecognized member to the system of normal human plasma glycoproteins: BJC1. Its plasma concentration appears to parallel those of total glycoproteins and α_1 -acid glycoprotein under conditions of trauma, neoplasm, and inflammation. Relatively low molecular weight probably accounts for its frequent appearance in the urine in these clinical situations.

BJC2 appears to contain the same protein moiety as BJC1 (as evidenced by amino acid composition and antigenic behavior) but a smaller complement of carbohydrate. By immunoelectrophoresis, this glycoprotein was visualized only in a small proportion of urines from cancer patients. Whether BJC2 is unique to neoplastic disease can be determined only after a potent and specific antiserum to this substance is developed.

We recently found (19) that plasma of patients with cancer frequently contains a glycoprotein with the same protein moiety as α_1 -acid glycoprotein but subnormal carbohydrate content. The relation between BJC1 and BJC2 may be analogous to that between normal α_1 -acid glycoprotein and the abnormal form found in the plasma of patients with cancer.

Among recently identified cancer-related proteins, carcinoembryonic antigen (CEA), like BJC1, contains more than 40% carbohydrate (20). However, the following differences show BJC1 and BJC2 are unrelated to CEA: BJC1 contains less than 0.026% of this antigen by radioimmunoassay; and amino acid composition of CEA differs¹⁰ from that of BJC1 and BJC2 with regard to 13 of 17 residues.

⁷ Since only BJC1 was visible in these plasmas by immunoelectrophoresis, the calculation of glycoprotein concentration was based on this substance as reference standard. If a portion of the plasma's immunoreactivity was due to BJC2, these calculations are low by a factor of 1 to 2.5.

⁸ Calculated values will be up to 2.5 times greater if the WBC contains BJC2 as well as BJC1.

⁹ Different because none reacted with rabbit antiserum to human plasma.

¹⁰ Recognized plasma proteins with known amino acid composition containing > 5% carbohydrate (α_2 -neuraminoglycoprotein, 43%; α_1 -acid glycoprotein, 41%; α_1 -X glycoprotein, 23%; haptoglobin, 20%; Zn- α_2 -glycoprotein, 18%; hemopexin, 17%; α_2 -glycoprotein, 17%; transcortin, 14%; tryptophan-poor α_1 -glycoprotein, 14%; β_2 E globulin, 14%; α_1 -antitrypsin, 13%; α_1 -easily precipitable glycoprotein, 13%; α_2 -HS-glycoprotein, 13%; immunoglobulins A, D, E, and M, 8–12%; 4.6S-postalbumin, 10%; inter α -trypsin inhibitor, 9%; ceruloplasmin, 8%; α_2 -macroglobulin, 8%; transferrin, 6%; fibrinogen, 5%) were compared with BJC1. In each case, content of five or more amino acids (expressed as residues per 1,000 residues) was > 2 SD removed from average values of BJC1 shown in Table II.

Immunodiffusion showed material reactive with anti-serum to BJC1 in extracts of leukemic but not of normal WBC. Only BJC1 was visualized in the leukemic extracts by immunoelectrophoresis. However, threshold concentration for detection by immunoelectrophoresis is probably higher for BJC2 than BJC1, because of the lesser reactivity of the former with the available antisera (Fig. 6).

What factors might explain the presence of anti-BJC1-reactive material in plasma of both normal and leukemic individuals, but in WBC of only the latter? Experiments on effect of hepatectomy on incorporation of radioactive monosaccharide into circulating glycoproteins of normal and tumor-bearing rats have shown (21, 22) that in the normal animal, plasma glycoproteins are made exclusively in the liver, and that in the tumor-bearing rat, a portion of these glycoproteins arises in extrahepatic, presumably neoplastic tissue. Probably BJC1, like other components of the plasma glycoprotein system, is normally synthesized in liver and released into plasma. In leukemia and perhaps other neoplastic conditions, the glycoprotein may also be synthesized by malignant cells. Alternatively, BJC1 or BJC2 might be adsorbed from plasma onto the surface of the leukemic, but not normal, WBC. Whether or not such adsorption occurs might relate to known differences in surface structure of leukemic versus normal cells (23).

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