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Research Article

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A Circulating Anticoagulant in γ_{1A}-Multiple Myeloma: Its Modification by Penicillin *

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This report is concerned with the presence of an inhibitor of antihemophiliac globulin (AHG, VIII) in a patient with multiple myeloma. Although inhibitors against AHG have been described in a variety of disorders (1–4), to our knowledge they have not been described in myeloma.

The inhibitory capacity was confined to those fractions of the patient's plasma that contained γ_{1A}-protein and was lost when the myeloma protein was adsorbed from the plasma by specific antiserum. Penicillin *in vivo* and penicillamine *in vitro* reversed the inhibition.

Methods

Samples were collected in 0.1 M sodium citrate, 1 vol of anticoagulant being used for each 9 vol of blood.

Clotting methods. Bleeding time was measured by the Ivy method (5), clotting time by the 3 tube Lee-White method in glass tubes (6). Platelets were enumerated by the method of Dameshek (7). The one-stage prothrombin was determined by the Quick assay (8), with rabbit brain thromboplastin.[1] In one instance the thromboplastin was serially diluted 1/10, 1/100, and 1/1,000 with saline, and the prothrombin time then determined with the diluted thromboplastin. Prothrombin content was determined by the *p*-toluensulfonyl-*l*-arginine methyl ester (TAMe) synthetic substrate method (9) and prothrombin consumption by a modification of the method of Sussman, Cohen, and Gittler (10). Exactly 1 hour after coagulation had occurred, the further conversion of prothrombin to thrombin was arrested by the addition of 0.2 ml of sodium citrate. The clot was disrupted, the tube centrifuged, and the test completed as described by the authors. The thrombin time was determined by methods previously described in this laboratory (11). Factor V was measured by the method of Stefanini and Dameshek (12), and Factor VII was measured by comparing the corrective effect of the patient's plasma and a normal plasma on that of a known VII-deficient plasma (13). Fibrinogen was measured by the method of Ratnoff and Menzie (14). The partial thromboplastin time (PTT) was determined by the procedure of Rodman, Barrow, and Graham (15) with the platelet substitute of Bell and Alton (16). It was utilized for special purposes as follows: *a*) The ability of the patient's plasma to correct plasmas with known deficiencies of Factors VIII, IX, and XI was determined. *b*) To assess the time dependency of the inhibitor, various amounts of the patient's and of normal plasma were incubated either separately and then combined, or together in identical proportions for 1 hour at 37° C (1, 2). After incubation the PTT was performed as usual. Longer periods of incubation were unreliable due to shortening of all the values. c) The PTT was likewise utilized to test for "contact factor." Plasma deficient in contact factor (XI, XII) was prepared with 20 mg of Celite per ml of plasma, and testing was performed by the method of Nossell (17). To the synthetic plasma was added either normal, noncontacted plasma or an identical volume of the fractions isolated by continuous flow electrophoresis (see below).

The thromboplastin generation test (TGT) was performed by the method of Biggs and Douglas (18) with citrate plasma adsorbed with aluminum hydroxide. Unless otherwise specified, the platelet substitute of Bell and Alton (16) diluted 1/100 with saline was utilized. Modifications of the test are described below.

A) To assess the effect of time on the inhibitory effect of the patient's plasma on a normal plasma, the patient's adsorbed plasma, normal adsorbed plasma, and mixtures thereof were incubated either separately or together as described for the PTT. After incubation the mixtures were diluted 1 to 5 with saline, and the testing was completed as usual (1).

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[1] Difco, Detroit, Mich.

B) The test was run without dilution of plasma or serum in an attempt to increase the inhibitory activity of the patient's plasma (19).

C) The patient's plasma served as the substrate plasma in the TGT, the other components being obtained from a normal subject.

D) To test for inhibition of thromboplastin in its "nascent" form, portions of normal or patient's adsorbed plasma were added to the generating mixture of a normal system at the 6-minute interval when thromboplastin production had reached its peak. The test was then continued for an additional 6 minutes.

E) To determine the inhibitory effect of isolated fractions, the patient's plasma was fractionated into 28 fractions by the continuous flow electrophoresis method of Lewis, Walters, Didisheim, and Merchant (20, 21). For testing, a single normal donor served as the control, and the same hemophiliac plasma was used throughout. Absorbed normal plasma diluted with saline to contain 0.03, 0.015 and 0.0075 ml of plasma per ml, respectively, served as the control. After 15 minutes of incubation 0.3 ml of each dilution was added to 0.3 ml of the adsorbed, AHG-deficient, nondiluted plasma. The test was completed by the addition of 0.3 ml normal serum 1/10, platelet substitute, and 0.02 M calcium. A standard curve was constructed for each day's experiments. Fractions isolated from the myeloma plasma were first diluted 1 to 5 with saline. To each 0.07 ml of the diluted fractions was added 0.03 ml of normal adsorbed plasma. The remainder of the test was performed as above. The 6- and 8-minute values for both test and control were averaged. The values were expressed as per cent inhibition of AHG activity of the normal sample calculated from each day's standard curve. When the values obtained with a plasma concentration of 0.03 ml per ml were considered as 100%, the standard deviation was ± 6%; similarly, for a plasma concentration of 0.015 ml per ml, the standard deviation was ± 5%. Values were considered significant if they exceeded the control by 2 SD or more.

F) To determine the effect of penicillamine on the TGT, penicillamine (3 mg per ml in saline) was added to equal volumes of adsorbed normal or patient plasma. The plasma-penicillamine mixtures were incubated either together at 37° for 60 minutes, or combined after incubation as previously described. After incubation the plasma-penicillamine mixtures were then diluted 1 to 5 with saline, and the TGT was completed as usual.

G) To determine the effect of penicillamine on the inhibitory capacity of myeloma plasma fractions, fractions shown to contain inhibitory effect were first pooled. The pooled fractions were preincubated for 60 minutes at 37° with saline (control) or an equal volume of saline containing 3 mg per ml of penicillamine. One vol of normal adsorbed plasma was then added to 4 vol of each mixture, and incubation continued for an additional 30 minutes. The TGT was then completed without further dilution of the plasma-fraction mixtures by the addition of normal serum 1/10, platelet substitute, and 0.02 M calcium.

Protein analysis, protein separation, immunoelectrophoresis, and antibody preparation. Paper electrophoresis was performed in barbital buffer, pH 8.6, with a current of 5 ma for 16 hours. Fractions used for determination of inhibitory activity were separated by continuous flow electrophoresis at 4° C by the method of Lewis and co-workers (20, 21) with a Spinco instrument. Two separate samples of plasma were run in this manner. Fractions eluted from the curtain were pooled and concentrated by pervaporation. After dialysis against 0.15 M saline for 48 hours, each fraction was made up to a final volume of 2 ml with saline. Each individual fraction was tested by immunoelectrophoretic analysis, and the amount of protein determined by a microbiuret method (22). The total recovery of serum globulin from the curtain on the second run was 86%. In addition starch block electrophoresis was performed by the method of Kunkel (23) on two other samples of the patient's plasma.

Ultracentrifugation [2] of the plasma was performed in a Spinco model E ultracentrifuge. The serum was diluted 1/5 in 0.24 M KCL buffered at pH 7.3 with minimal KPO_4. The samples were centrifuged at 50,740 rpm at 20° C.

Microimmunoelectrophoretic analysis of sera and protein fractions were tested by microimmunoelectrophoretic analysis according to the method of Scheidegger (24) as modified in our laboratory (25). The supporting medium was 2% Noble agar in barbital buffer pH 8.6 (ionic strength = 0.05).

Quantitation of serum proteins. The myeloma protein and serum immunoglobulin levels were quantitated by an immunoelectrophoretic-precipitin method (25). Gamma$_2$-globulin serum levels were expressed in grams per 100 ml and the γ_{1A} and γ_{1M} levels in units per milliliter [3] as well as in milligrams per milliliter.

Antisera. Antiserum specific for γ_{1A} was prepared in rabbits by the injection of highly enriched γ_{1A} preparations obtained from human colostrum (26).

Gamma$_2$-globulin prepared by DEAE cellulose chromatography (27) and small amounts of serum from a patient with no detectable γ_{1A} were used to adsorb cross-reacting antibodies. The adsorbed antiserum showed only a single arc typical of γ_{1A} when tested against human serum by immunoelectrophoretic analysis.

Removal of the myeloma protein. The γ_{1A}-myeloma protein was removed from the patient's plasma by the addition of rabbit antiserum specific for γ_{1A} (see above). Quantitation of the γ_{1A} levels of the samples before and after removal by the antiserum was performed by the immunoelectrophoretic-precipitin method. Since the rabbit antiserum itself showed an accelerating effect on a normal TGT, it was heated at 56° C for 30 minutes, centrifuged, and absorbed with 50 mg per ml of barium carbonate before use.

Reduction of serum samples. The effect of reducing agents on the serum immunoelectrophoretic patterns of the patient was tested by the addition of either 2-mercap-

[2] We are indebted to Dr. Eugene Conway of the Cincinnati Veterans Hospital for these studies.

[3] One U of γ_{1A} equals approximately 0.2 mg of protein.

toethanol or penicillamine or both to the serum. 2-Mercaptoethanol was added to the patient's serum to a final concentration of 0.2 moles per L, and the mixture was incubated for 1 hour at room temperature. The serum was then tested by immunoelectrophoretic analysis. Six hundred twenty-five μg of penicillamine in 0.01 ml of barbital buffer (pH 8.6, ionic strength 2 =.05) was added to 0.1 ml of serum and the mixture incubated for 1 hour at 37° C; the serum was then tested by immunoelectrophoretic analysis. The sera that were reduced were compared with other serum samples to which equivalent amounts of buffer only were added.

Other laboratory tests. The Sia water test was performed as described by Stefanini and Dameshek (12). Cryoglobulins were determined by collecting the sample in a warm syringe, allowing the sample to clot at 37°, separating the serum, and cooling at 4° C for 24 hours. Cryofibrinogens were determined by previously described methods (28). Fibrinolytic activity of the patient's plasma was measured on unheated plates by a modification of the method of Astrup and Mullertz (29).

Case history. L. N. (CGH 391-364), a 70-year-old Negro male, was first seen on the dermatologic service because of an eczematoid exfoliative dermatitis, which had been present intermittently for 20 years. His only hospitalization had occurred on the dermatology service. He denied backache, pain, weakness, fatigue, or weight loss. No abnormalities other than the dermatitis were detected upon physical examination. The laboratory studies were as follows: the hematocrit was 31%; the leukocyte count was 6,300; the differential count was normal; and platelets appeared adequate on smear. The corrected sedimentation rate was 3 mm per hour. The urine culture was normal, no protein was detected, and Bence Jones protein was absent. Culture of the urine was sterile, and no cells were noted in the voided specimen. The blood urea nitrogen (BUN) was 8 mg per 100 ml, and fasting blood sugar (FBS) was 76 mg per 100 ml. The Kahn and the Venereal Disease Research Laboratories syphilis tests were both negative, as were the cephalin flocculation, thymol, and zinc turbidity tests. The total protein was 8.9 g per 100 ml. Paper electrophoresis showed 30% albumin, 0.52% α_1-, 1.01% α_2-, 54.4% β-, and 13.6% γ-globulins. The myeloma protein was present in a narrow M peak in the beta region. By immunoelectrophoretic analysis, the level of γ_{1A} was 460 U, more than 20 times the normal adult level. Gamma$_2$-globulin was 500 mg per 100 ml, and γ_{1M} was 1.3 U per ml (normal, 6 to 20 U per ml). Bone marrow aspiration revealed marked plasmacytosis of approximately 70%. Many immature forms were present. Skin biopsy revealed only dermatitis. No definite lytic lesion or osteoporosis was noted in the spine, ribs, or pelvis. A questionable lytic lesion was seen on skull films.

The coagulation studies performed at this time, although incomplete, were normal. The clotting time was 10 minutes (normal, < 15 minutes), with normal clot retraction. The one-stage prothrombin time was 12 seconds (100%), and the prothrombin consumption was 41 seconds (normal, > 20). The thrombin time was also normal, i.e., 12 seconds. Treatment was limited to oral and parenteral steroids for a 3-week period, with improvement of skin lesions.

The patient left the hospital and was not seen for an 18-month interval. During this period there was a 50-pound weight loss accompanied by backache, fatigue, and weakness. On readmission to the medical service of the hospital the cutaneous lesions were still present. No adenopathy or splenomegaly were noted. Films of the skull and lumbar vertebrae now revealed lytic lesions and some degree of osteoporosis. Albumin was present in the urine, but Bence Jones protein was not detected. The sedimentation rate was 17 at room temperature. Plasma and serum calcium were both 9.1 mg per 100 ml (11), phosphorus 3.4, BUN 9, and FBS 79, mg per 100 ml. The red-blood cell count was 2,600,000, the hemoglobin (Hb) was 6.4 g, and the hematocrit was 26. The total protein was 8.9, of which 11.4% was albumin, and the remainder globulin, partitioned as follows: 0.79% α_1-, 2.36% α_2-, 81% β-, and 3.9% γ-globulin. The narrow M peak was again seen on paper electrophoresis in the area of the beta protein. Proteinuria was detected, but Bence Jones protein was absent. Paper electrophoresis of the urine revealed two bands, one in the albumin area, a second in the beta zone.

The clotting studies, in contrast to those obtained 18 months earlier, were definitely abnormal. Clot retraction was only fair in spite of an adequate platelet count. The Sia water test was positive, cryoglobulins were present in his serum, but cryofibrinogens were not observed (28). A skin biopsy again revealed the nonspecific changes of dermatitis. The biopsy site bled for 5 days after excision, but aside from large cutaneous purpuric lesions and retinal hemorrhages, no other abnormal bleeding was or has ever been noted. During this period the patient developed pneumonia, for which he received penicillin. Subsequent improvement in his clotting studies suggested a relationship to the penicillin therapy. Cyclophosphamide (cytoxan) therapy was commenced on the eighth day of admission to the medical service and continued for a total of 11 months.

Initially the patient showed improvement with some regression of the bone lesions, weight gain, and increase in strength; however, there was no associated change in the serum protein patterns nor improvement of the anemia. For the last 5 months his clinical condition has deteriorated.

Results

Initial studies. The clotting studies when the patient was first admitted to the medical service in 1962 are presented in Table I. Moderate abnormalities were noted in the one-stage prothrombin time, prothrombin content as measured by TAMe, and Factors V and VII. When rabbit brain thromboplastin was serially diluted and then used to determine the prothrombin times, those of the patient were no more prolonged than the controls,

TABLE I

Initial studies when clotting abnormalities were first detected

	Patient	Control	Normal values (range)
1. Prothrombin time (one stage), *seconds*	15	12	12–14
2. Prothrombin, TAMe* *U/ml*	39		53 ± 5
3. Factor V, *% of normal*	40	120	60–160
4. Factor VII, *% of normal*	55		50–150
5. Prothrombin time, *seconds*, tissue thromboplastin was diluted 1/10	19	16	
6. Prothrombin time, *seconds*, tissue thromboplastin was diluted 1/100	28	28	
7. Prothrombin time, *seconds*, tissue thromboplastin was diluted 1/1,000	51	49	
8. Thrombin time, *seconds*†	19	14	11–14
9. Thrombin time, plus 0.02 M $CaCl_2$, *seconds*	14	10	
10. Bleeding time, *minutes*	5		< 5
11. Clot retraction	Fair	Good	
12. Platelets, 10^{-5}	230		>200
13. Fibrinogen, *mg/100 ml*	184		250–350
14. Clotting time, 3 tube Lee-White, *minutes*	31		< 15
15. Prothrombin consumption, *seconds*	12		> 20
16. Partial thromboplastin time, *seconds*	122	65	60–120
17. Thromboplastin generation clot time (6 minutes activation), *seconds*	27	9	8–11
18. Proteolysis, fibrin plate	None	None	

* Determined by the *p*-toluensulfonyl-*l*-arginine methyl ester (TAMe) synthetic substrate method.
† Thrombin time of mixtures: for 20 parts patient's plasma (P) to 80 parts normal plasma, 17 seconds; for 50 P to 50 normal, 16 seconds.

thus demonstrating the lack of inhibitors against the action of tissue thromboplastin (1). The thrombin time was prolonged, indicating a defect in, or inhibition of, fibrinogen conversion. Both 20 and 50% of the patient's plasma had a slight but definite inhibitory effect on the thrombin time of a normal plasma (Table I). However, in contrast to the findings previously reported from this laboratory, the addition of 0.02 M $CaCl_2$ did not shorten the thrombin time of the patient's plasma to a greater degree than that of the normal plasma. Abnormal calcium binding by the myeloma protein with differences between plasma and serum values were not observed, both plasma and serum Ca^{++} being 9.1 mg per 100 ml (11). The bleeding time was normal, clot retraction was only fair in spite of an adequate number of platelets, and fibrinogen was somewhat low.

Studies on the site and nature of the inhibitor in the first stage of clotting. The most marked changes were noted in the first stage of clotting, i.e., intrinsic thromboplastin production, as indicated by a prolonged clotting time, abnormalities in prothrombin consumption, PTT, and TGT. (Tables I and II). No defect could be found in the patient's platelets. The abnormality was corrected by substitution of normal plasma, suggesting a defect in or block of the availability of AHG (VIII). Substitution of normal serum had no corrective effect on the patient's TGT. When the TGT was run without dilution of plasma and serum, the test showed improvement in contrast to the usual inhibitors of AHG (VIII) (19). The patient's serum, when incubated with normal plasma, did not appear to contain any inhibiting substance, an observation previously noted with Factor VIII inhibitors when unadsorbed serum was used for testing in the TGT (30).

The patient's plasma had no corrective effect in the PTT when tested against a plasma known to be defective in AHG (VIII), but it corrected the defects of known plasma thromboplastin component (PTC) (IX) and plasma thromboplastin antecedent (PTA) (XI)-deficient plasmas (Table III). The corrective effect of the patient's plasma against these two deficient plasmas suggested that no block existed in the activation of PTC by the contact factor, nor in the interaction between activated PTC and AHG at a later stage in thromboplastin production.

A slight delay in fibrinogen conversion (thrombin time) was a consistent finding (Table I, no.

TABLE II

Thromboplastin generation tests, initial observations

Contents of activation mixture	Activation time (minutes) 4	6	8	10	Interpretation
	Clotting time				
	seconds				
1. N. Plas., N.S., N. Plat.*	11	12	10	10	Control
2. P. Plas., P.S., P. Plat.	55	33	20	20	Patient abnormal
3. N. Plas., N.S., P. Plat.	13	13	13	14	Patient's platelets normal
4. N. Plas., N.S., Plat. sub.	9	9	10	10	Control, platelet substitute
5. P. Plas., P.S., Plat. sub.	30	27	29	30	Patient with platelet substitute, abnormal
6. N. Plas., N.S., Plat. sub., P. Plas. as substrate	13	12	13	13	Abnormalities in TGT† not due to defect in stage 3
7. N. Plas., P.S., Plat. sub.	10	10	11	12	Normal plasma corrects, therefore AHG‡ (VIII)-like defect
8. P. Plas., N.S., Plat. sub.	73	70	56	25	No correction with normal serum, therefore no PTC§ (IX) defect
9. N. Plas., N.S., undiluted, Plat. sub.	11	11	11	12	Control same, diluted (no. 4), or undiluted
10. P. Plas., P.S., undiluted, Plat. sub.	36	15	12	15	Patient improves without dilution (no. 5)
11. N. Plas., N.S., incubated 30 minutes at 37°, diluted, tested	10	11	12	13	Normal serum does not inhibit normal plasma
12. N. Plas., P.S., incubated 30 minutes at 37°, diluted, tested	12	12	12	13	Patient's serum does not inhibit normal plasma

* N = normal; P = patient; Plas. = plasma; S = serum; Plat. = platelet; Plat. sub. = platelet substitute. In no. 1 through 8, plasma was diluted 1/5, serum, 1/10. In no. 9 and 10, plasma and serum were undiluted. In 11 and 12, 1 vol of adsorbed plasma and ½ vol of serum were incubated together; the mixture was then diluted 1/5 and testing was completed as usual.

† TGT = thromboplastin generation test.

‡ AHG = antihemophiliac globulin.

§ PTC = plasma thromboplastin component.

8). Since all methods of coagulation are based on the appearance time of the fibrin clot, it was important to determine if this was of significant magnitude to influence the assays in an earlier stage of clotting. When the patient's plasma served as the substrate or indicator plasma for the TGT performed with plasma and serum derived from a normal subject, no inhibition was observed (Table II, no. 6). Fibrin monomers resulting from the partial proteolysis of fibrinogen may likewise delay the rate of fibrinogen conversion (31, 32). Proteolysis was not demonstrated when tested on two occasions (Table I, no. 18). Finally, the amount of calcium utilized in the TGT and PTT was of significant concentration to nullify the defect of fibrinogen conversion as seen in myeloma

TABLE III

Effect of myeloma plasma on plasmas with known deficiencies of AHG (VIII), PTC (IX), and PTA (XI), as determined by partial thromboplastin time†*

Date	Addition of	Amount	Deficient plasma AHG (180)	PTC (200)	PTA (330)
			seconds		
11/15/62	Normal (64)	20/80	88		170
		50/50	60	98	118
	Myeloma (122)	20/80	152		160
		50/50	130	95	120
			AHG (224)		
3/31/64	Normal (90)	50/50	95		
	Myeloma (148)	50/50	137		

* PTA = plasma thromboplastin antecedent.

† Normal or myeloma plasma was added to plasmas with proven deficiencies of Factors VIII, IX, or XI, and the partial thromboplastin time (PTT) of the mixtures was determined. In column 3, the numerator represents the quantity of normal or myeloma plasma, the denominator the amount of the deficient plasma in each mixture. Figures in parentheses represent the PTT in seconds of 100% of each individual plasma.

TABLE IV

Effect of incubation at 37° for 30 minutes on the inhibitor, myeloma plasma

Contents	Thromboplastin generation Clotting time after 6 minutes activation				Partial thromboplastin time Clotting time			
	10/4/62		3/20/64		10/4/62		3/26/64	
	Separately	Together	Separately	Together	Separately	Together	Separately	Together
	seconds				*seconds*			
N (normal plasma) 100% (control)	12		11		88		120	
P (myeloma plasma) 100%	19		48		147		321	
20 N/80 P	12	15	42	32	113	112	200	185
50 N/50 P			17	19	107	103	160	140
80 N/20 P	11	11	14	13			160	180

plasma (11) (Table I, no. 9). All of these observations indicated, therefore, that the major defect was present at an earlier stage of clotting.

The patient's plasma was unable to inhibit "intrinsic thromboplastin" once it had formed. The TGT was performed in the usual manner with plasma, serum, and "indicator" plasma obtained from a normal subject. At the peak of thromboplastin production, after 6 minutes of activation, an equal volume of the patient's adsorbed plasma was added to the generating mixture and testing continued for an additional 6 minutes. The values at 8, 10, and 12 minutes' activation were 11, 12, and 13 seconds, compared with 11, 12, and 12 seconds when normal plasma was similarly utilized.

Effect of incubation. Incubation of the patient's plasma with normal plasma did not accentuate the defect (Table IV), in contrast to the findings observed with the usual inhibitors of Factor VIII (1–3). In the TGT no further changes were observed after a 2-hour period of incubation. Incubation in the PTT was unsatisfactory for longer than 1 hour since even normal citrate plasma showed some shortening on incubation.

The presence of an inhibitor rather than a simple defect was suggested by the findings in Table V.

TABLE V

*Amounts of normal plasma required to correct the defect in myeloma and AHG (VIII)-deficient plasmas**

Date	Addition of	Amount		
			seconds	
	A) Thromboplastin generation, clotting time after 6 minutes activation			
			Myeloma (19)	VIII
10/ 4/62	Normal (12)	20/80	11	
			Myeloma (48)	VIII (34)
3/20/64	Normal (9)	10/90		12
		20/80	31	10
		50/50	19	
		80/20	14	
	B) Partial thromboplastin time			
			Myeloma (147)	VIII (167)
10/ 4/62	Normal (88)	10/90	145	93
		20/80	107	88
		30/70	113	
			Myeloma (320)	VIII (237)
3/26/64	Normal (85)	10/90		135
		20/80	200	88
		50/50	165	85
		80/20	160	

* Normal plasma was added either to the myeloma plasma, or to that with a proven deficiency of Factor VIII. In column 3, the numerator represents the amount of normal plasma added to the myeloma or VIII-deficient plasma, the denominator the amounts of myeloma or VIII-deficient plasma in the mixture. Figures in parentheses represent the values for 100% of each individual plasma.

The large amounts of normal plasma needed to correct the defect in the patient's plasma on March 20, 1964, are characteristic of inhibitors rather than a simple defect of Factor VIII. When normal plasma was used to correct the defect in a Factor VIII-deficient plasma, much smaller quantities were required (Table V). As likewise shown, the amounts of normal plasma needed to correct the defect in the patient's plasma varied with the severity of the coagulation disorder at the time of testing.

Additional studies on the nature of the inhibitor. The inhibiting substance in the patient's plasma was not removed by a variety of adsorbents, including aluminum hydroxide, barium carbonate, and barium sulfate. Its activity was unimpaired by dialysis against saline, by heating at 37° for 4 hours, 56° for 30 minutes, or freezing at − 20° for 6 months. The addition of 0.3 ml of protamine [4] in the TGT had no corrective effect, indicating that the inhibitor was not heparin or a heparin-like substance.

Changes noted with penicillin in vivo and penicillamine in vitro. The corrective effect of *in vivo* penicillin was observed by chance when the patient was being treated for pneumonia. Improvement was demonstrated by a variety of procedures, including shortening of the clotting time, normalization of the thrombin time (TT) and PTT and improvement in the TGT (Table VI). Large doses of penicillin of 8,000,000 U or more given either orally or parenterally were required to produce these changes. The duration of the effect was still partially manifest for 16 hours after completion of the penicillin therapy (Table VI).

Plasma obtained from the patient preceding the

[4] One per cent, diluted 10/3 to 10/8. These amounts of protamine had no effects on the normal TGT.

TABLE VI

Changes in coagulation in myeloma before (B) and after (A) penicillin

Date	Clotting time		Prothrombin consumption		Thrombin time		PTT		Thromboplastin generation activation time (minutes)								Penicillin dose, type, and testing interval
									B				A				
									4	6	8	10	4	5	8	10	
	B	A	B	A	B	A	B	A	Clotting time								
	minutes		*seconds*		*seconds*		*seconds*		*seconds*								
10/ 4/62	22	13			19	12	147	53	39	19	16	18	29	13	13	16	8,000,000 U oral in 24 hours; tested 3 hours after last dose.
10/12/62	21	15	15	26					<60	41	16	15	50	17	12	12	10,000,000 U aqueous iv in 6 hours; tested 3 hours after completion of prescription.
10/24/64			17	25	17	14			<60	<60	59	28	60	60	29	12	10,000,000 U iv in 6 hours; tested at end of infusion.
11/13/62									73	42	17	16	41	20	14	13	15,000,000 U iv in 12 hours; tested 16 hours after completion of prescription.
11/17/62							210						70	70	60	24	600,000 U procaine im every 12 hours for two doses; tested 3 hours after last dose.
Normal or control values	<15		>20		11–14		60–120		12	10	10	11*					

* Mean of 5 controls run concomitantly with tests.

TABLE VII

*Inability of myeloma plasma to correct AHG (VIII)-deficient plasma, with correction thereof after in vivo penicillin; determined by PTT**

	Before penicillin	After penicillin
	seconds	
Normal	74	60
Patient	147	53
Hemophiliac	139	150

Addition of normal or patient's plasma to hemophiliac plasma

Per cent added	Normal	Patient	Normal	Patient
10	94	144	72	87
20	88	152	57	56
30	78	135		

* Various amounts of normal plasma and the myeloma plasma were added to that of a known hemophiliac, and the PTT was determined. The patient's plasma was obtained preceding and 4 hours after 8,000,000 U of oral penicillin given over a 24-hour period.

penicillin therapy had no corrective effect on that of a known hemophiliac. After penicillin therapy, however, the patient's plasma was as effective as the normal plasma in correcting the defect of an AHG plasma, again suggesting no defect in factor VIII but, rather, interference with its availability (Table VII). It appears unlikely that the changes after penicillin were fortuitous (Table VIII). From 1962 on, samples tested on 13 different occasions were all abnormal with one exception (1/3/62). These normal findings were observed approximately 2½ months after commencing cytoxan therapy, and they occurred during a period of clinical improvement as manifested by gain in weight and regression of the osteolytic lesions. With the recurrence of symptoms and progression of illness the clotting defects returned.

The changes in the clotting mechanism after penicillin therapy were accompanied by the appearance of a new precipitation arc in the beta area as shown by immunoelectrophoretic analysis. Similar arcs were noted upon *in vitro* treatment of the serum with 2-mercaptoethanol and penicillamine (Figure 1). However, the serum level of the γ_{1A} as measured by the immunoelectrophoretic precipitin technique did not fall after penicillin therapy.

Correction of the abnormal TGT was demonstrated with penicillamine (Table IX). Results obtained on incubation of the plasma with penicillamine were only moderately shorter than those obtained on immediate combination, indicating the rapid rate of the reaction. Penicillin itself had no corrective effect. Large amounts inhibited the TGT of the normal system, whereas small amounts were ineffective.

Removal of myeloma by specific antiserum. Rabbit antiserum against γ_{1A}-globulin was prepared as previously described (26). This anti-

TABLE VIII

Coagulation studies, myeloma at intervals when penicillin was not administered

Date	Clotting time*	Prothrombin consumption†	Thrombin time		PTT		Thromboplastin generation clotting time after 6 minutes activation	
			Patient	Control	Patient	Control	Patient	Control
	minutes	*seconds*	*seconds*		*seconds*		*seconds*	
3/16/61	13	41	10	9	100	72		
10/ 2/62	31	12	19	14	146	86	27	10
10/ 3/62					147	74	20	11
10/ 8/62	35	15	20	12	212	113	64	12
10/10/62‡	21		22	14			60	14
10/25/62	24	14						
11/ 6/62							52	12
11/11/62	27						60	10
11/19/62							86	12
12/20/62							60	11
12/28/62							31	11
1/ 3/63	20	46					11	10
1/17/63			12	13			14	11
3/13/64		16			350	110	29	8

* Normal = <15 minutes.
† Normal = >20 seconds.
‡ Cytoxan therapy 10/9/62 thru 10/31/63; drug A20968 11/27/63 to present.

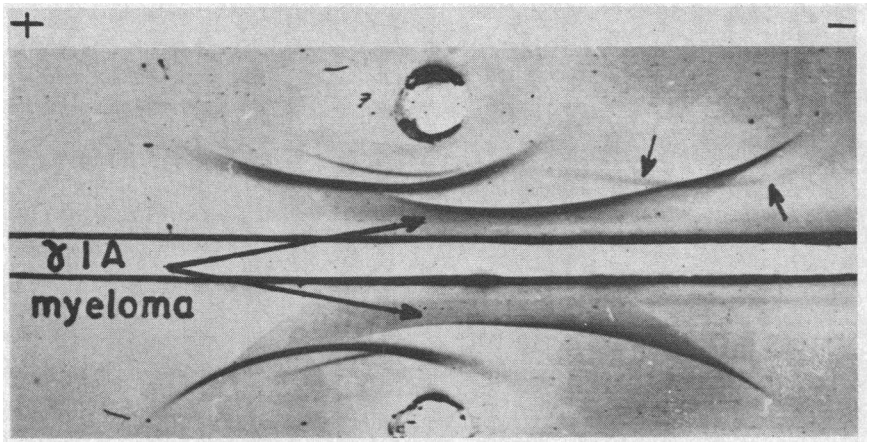

FIG. 1. IMMUNOELECTROPHORETIC PATTERN OF MYELOMA PLASMA BEFORE AND AFTER TREATMENT WITH REDUCING AGENTS (SEE RESULTS SECTION OF TEXT). Below, before treatment; above, after treatment. Pattern developed by antiserum to serum proteins adsorbed with normal cord blood. Arrows indicate new precipitin arc. A similar pattern was noted with *in vivo* penicillin.

serum was found to have an accelerating effect on the TGT of a normal system. However, when first adsorbed with barium carbonate (50 mg per ml) and then heated (56° C for 30 minutes), the accelerating effect disappeared. This heated adsorbed preparation had no corrective effect on the plasma of a known hemophiliac. Likewise, it showed no accelerating or inhibiting effect on the TGT of a normal system (Figure 2). When added in equal volume to the patient's plasma, incubated at 37° for 30 minutes, and then tested by the TGT, marked improvement was noted (Figure 2). As the antiserum was diluted, less correction was observed.

Studies with fractions isolated from the patient's plasma. The patient's plasma and a normal plasma were fractionated by starch block electrophoresis (23). Two pools (A) and (B) were made from two separate samples of the patient's plasma. As determined by immunoelectrophoresis, pool A contained 115 U per ml and pool B, 184 U per ml of the myeloma protein. Inhibition of the normal system was demonstrated in the PTT when the ratio of the abnormal protein was adjusted to approximate that of the parent plasma.[5] Under these conditions both pools A and B showed inhibition (Figure 3), and B was more active. Fractions obtained from a normal plasma had no

[5] The patient's plasma contained 460 U of γ_{1A}-globulin per ml.

TABLE IX

Effect of penicillamine on the thromboplastin generation test on myeloma and normal plasmas

Activation time	Myeloma plasma Incubated* 5/21/64 Separately +NaCl	Separately +Pen.	Together +NaCl	Together +Pen.	Myeloma plasma Incubated* 1/24/63 Together +NaCl	Together +Pen.	Normal plasma (control) Incubated 5/21/64 Separately +NaCl	Separately +Pen.	Together +NaCl	Together +Pen.
minutes	*seconds*				*seconds*		*seconds*			
4	54	52	55	24	59	50	37	37	39	34
6	39	18	29	15	29	18	13	14	17	13
8	28	16	15	11	23	15	10	10	11	10
10	16	12	12	12	24	14	10	10	12	10

* Aluminum hydroxide-adsorbed citrate plasma plus saline, or penicillamine in saline, were incubated for 60 minutes at 37° either separately and then combined, or together. After incubation the mixtures were diluted 1/5 with saline and the TGT was completed as usual. Controls of 1/23/63 similar to 5/21/64 have been omitted. The final activation mixture contained 0.075 mg penicillamine.

inhibitory effect. The patient's and normal plasmas were likewise fractionated by continuous flow electrophoresis, by the method of Lewis and co-workers (20, 21). The initial 10 ml of plasma was separated into 28 fractions. Each fraction was identified by immunoelectrophoretic analysis,[6] and its protein content determined by the microbiuret method (22). The corrective effect of normal plasma on that of a known hemophiliac was first determined employing the TGT. After incubation of each individual fraction with the normal plasma the corrective effect of the normal plasma was again tested. Studies with the preliminary run showed that the inhibitory activity was confined chiefly to those fractions containing the largest amount of γ_{1A}-protein. In the second run (Figure 4) not only was the total protein of each fraction determined but also the myeloma protein and serum immunoglobulin levels were quantitated by the immunoelectrophoretic-precipitin method.

[6] Fraction 4 was the first found to contain measurable protein, and is indicated on the graph in the number one position.

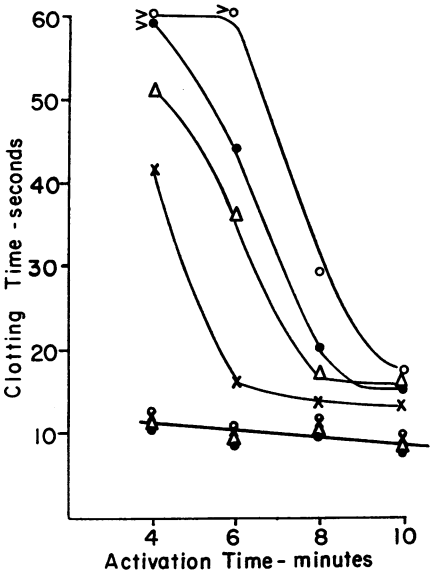

FIG. 2. EFFECT OF ADDITION OF SPECIFIC ANTI-γ_{1A}-ANTISERUM ON THE THROMBOPLASTIN GENERATION TEST (TGT) OF THE MYELOMA PLASMA. O——O myeloma plasma plus saline; ×——× myeloma plasma plus equal volume of undiluted specific anti-γ_{1A}-antiserum; △——△ myeloma plasma plus antiserum diluted 1/10; ●——● myeloma plasma plus antiserum diluted 1/20. The bottom line indicates normal plasma plus similar dilutions of the antiserum.

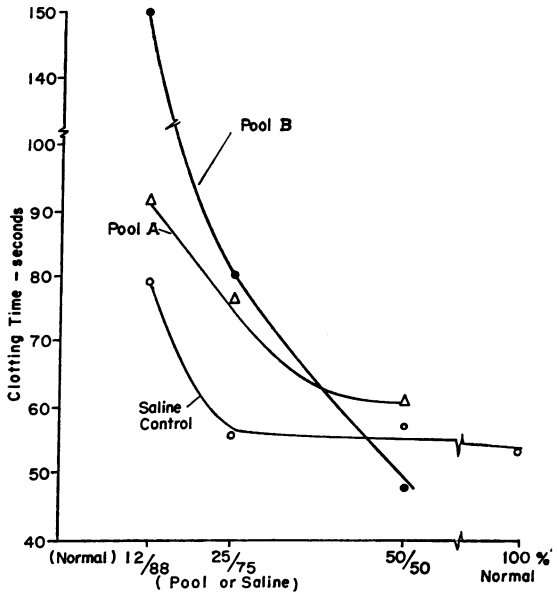

FIG. 3. BETA-MYELOMA PROTEIN (POOLS A AND B) ISOLATED FROM TWO SAMPLES OF PLASMA BY STARCH BLOCK ELECTROPHORESIS. Various amounts of the fractions and normal plasma were incubated for 30 minutes at 37° and then tested with the partial thromboplastin time. Normal plasma incubated with saline served as the control.

With one exception (tube 11) inhibition occurred only in those tubes containing considerable amounts of γ_{1A}-(beta) protein. The failure to demonstrate inhibition in this fraction, which contained large amounts of γ_{1A}-protein, was difficult to explain. It was found, however, that the fraction contained "contact factor," which, by altering the test system, may have obscured the inhibiting effect of the fraction itself. Synthetic contact factor-deficient plasma was made by the method of Nossell (17) and subsequently used for testing in the PTT. The clotting time of the deficient plasma was 175 seconds. No shortening was observed upon the addition of $\frac{1}{3}$ vol of either normal, noncontacted plasma, of fraction 6, or of fraction 9. The addition of a similar volume of fraction 11 shortened the PTT from 175 to 70 seconds. Normal plasma similarly tested showed no inhibition, whereas fractions 8 and 9 showed some contact factor activity (20, 21).

To determine whether the inhibition was due simply to a high protein concentration, purified human albumin[7] in concentrations equal to frac-

[7] Kindly donated by Dr. C. D. West, The Children's Hospital, Cincinnati, Ohio.

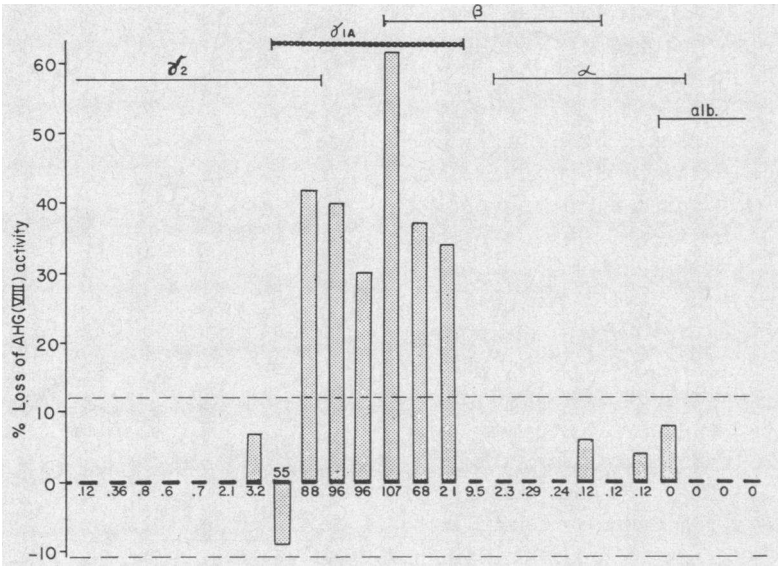

FIG. 4. MYELOMA PLASMA SEPARATED INTO 28 FRACTIONS BY CONTINUOUS FLOW ELECTROPHORESIS. The first fraction containing any measurable protein (no. 4) is shown on the graph in the first position. Fractions were preincubated with normal adsorbed plasma and the mixtures subsequently added to antihemophiliac globulin (AHG) (VIII)-deficient plasma. The TGT was used for testing (see Methods). Values have been expressed as per cent loss of AHG (VIII) activity. The numbers below the columns represent milligrams of γ_{1A}-protein per milliliter of the fractions. The dotted lines represent ± 2 SD.

tion 15, which contained the largest amount of γ_{1A}-protein, and purified γ_2-globulin [7] in similar concentrations, was added but did not inhibit the restorative effect of normal plasma on the AHG-deficient plasma.

The inhibiting effect of the fractions was not destroyed by heating at 37° for 4 hours, nor was it removed by a variety of adsorbents including barium carbonate, barium sulfate, and aluminum hydroxide.

Because of the scarcity of the material, aliquots of fractions 12 through 17 were pooled and the experiments performed as described in Methods (see thromboplastin generation test, *G*). The pooled fractions were found to inhibit the normal plasma utilized in the TGT. In the presence of the pool, the clotting time at the 6-minute activation period was prolonged to 21 seconds, compared with a clotting time of 11 seconds of normal plasma plus saline, or normal plasma plus penicillamine. When the pooled samples were first preincubated with penicillamine,[8] their inhibitory effect was reversed to 10 seconds at the 6-minute activation period. The data indicate that AHG (Factor VIII) was bound by the abnormal proteins in the patient's plasma so that it was no longer available. Still other evidence for the tendency of the γ_{1A}-protein to form complexes is seen in Figure 5, where alteration of the mobility of the albumin in the patient's serum is shown, apparently due to complex formation with the myeloma protein (33).

Discussion

Inhibitors of AHG are known to occur in certain types of patients. They are most often seen in hemophiliacs, particularly those who have received multiple transfusions (34–37), and in postpartum patients (38, 39). They have been observed in patients with penicillin reactions (1, 40), and in pemphigus (41). In a large number of subjects no basic disorder can be detected (1). These in-

[8] The final activation mixture contained 0.075 mg of penicillamine.

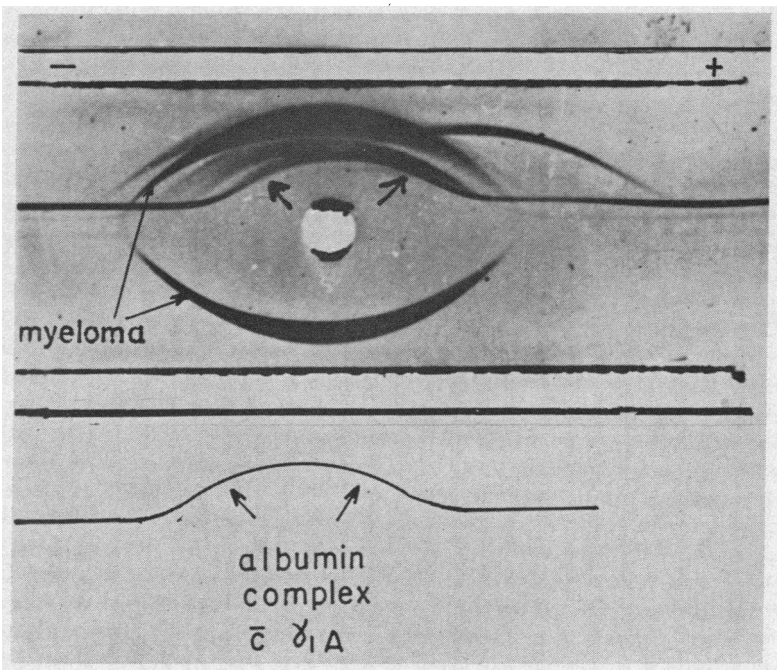

FIG. 5. SERUM ALBUMIN SHOWN IN AREA OF γ_{1A}-MYELOMA BY LINE OF IDENTITY. A fraction (no. 17) from continuous flow electrophoresis has been reacted against unadsorbed antinormal human serum (above) and specific anti-γ_{1A} prepared by adsorption of the above antiserum with serum from a patient having no detectable γ_{1A} (below). The linear arc is due to excess albumin in the adsorbed antiserum reacting with antialbumin diffusing from above. Complexed albumin with γ_{1A} mobility is shown by the arrows.

hibitors differ in several respects from those seen in our patient. They are found chiefly in the γ_2-globulin fraction of the plasma (42–44). Incubation of normal and pathologic plasmas together accentuates the defect as incubation is prolonged (1, 30). Finally, testing in undiluted systems accentuates rather than diminishes the usual AHG inhibitor (19).

Hemorrhagic tendencies, thrombocytopenia, and coagulation disorders are well known in myeloma (45, 46). The commonest disorder is inhibition of fibrinogen conversion by the offending protein (11, 47–49). However, we could find no report of inhibition of AHG as seen in our patient.

Beneficial effects of penicillin and penicillamine have been described, but only in Waldenström's macroglobulinemia. Ritzmann, Thurm, Truax, and Levin (50) described two patients with macroglobulinemia and clotting disorders including prolonged clotting times, abnormal prothrombin consumption, and an abnormal TGT with an AHG-like defect. In a subsequent article, Ritzmann, Coleman, and Levin (51) observed reversal in viscosity, increase in sedimentation rate, and changes in the electrophoresis in a patient with macroglobulinemia treated with either penicillamine or large doses of intravenous penicillin. Bloch, Prasad, Anastasi, and Briggs (52) noted changes in Waldenström's disease after penicillamine therapy. There was some decrease in the globulin and a change in the pattern of ultracentrifugation after the medication. The sulfhydryl content of the patient's plasma was low, and no such groups were found in the isolated macroglobulin. Long and co-workers (53) noted interference with prothrombin conversion and Factor V utilization in macroglobulinemia. We were unable to demonstrate excess macroglobulin in our patient either by ultracentrifugation of the serum or by the immunoelectrophoretic technique. Indeed, the level of γ_{1M} was low (1.33 U per ml) as compared with a normal of 6 to 20 U. These observations as well

as the appearance of the bone marrow, coupled with the clinical findings, confirmed the diagnosis of myeloma.

The data clearly indicate that the γ_{1A}-protein in some way interfered with the availability of the AHG. Whether the abnormal or small amounts of normal γ_{1A}, if present in the patient's serum at all, or both were at fault cannot be determined inasmuch as there is at present no means of separation of the paraprotein from the normal globulin. The other immunoglobulins, γ_2 and γ_{1M}, appear to be excluded from inhibitory activity since the degree of suppression paralleled the electrophoretic distribution of γ_{1A} and, most important, the capacity to inhibit was removed by elimination of the myeloma protein by a specific antiserum. The antiserum was rendered specific for γ_{1A} by removing cross-reacting antibodies by adsorbtion with γ_2-globulin.

The interaction of γ_{1A}-myeloma with albumin and haptoglobin has been demonstrated by Heremans (33) and was shown for albumin in the present case (Figure 5). The association with albumin and haptoglobin in complexes that appeared to be of high molecular weight and possessed electrophoretic mobility different from the normal protein as described by Heremans appeared to be labile to disulfide bond reduction and disappeared on treatment of the serum with 0.1 M cysteine. It was postulated that the albumin-myeloma interaction was mediated by disulfide bonding, the large number of free-SH groups in albumin favoring such association. However, it is unknown at present whether or not free-SH groups exist on γ_{1A}.[9] Since haptoglobin does not have any free-SH groups and the albumin-SH groups seem to be poorly reactive in the absence of denaturing agents (54), it seems more likely that protein association is dependent upon noncovalent interactions. In this event, the disruption of the complex by reducing agents could be attributed to conformational changes of the γ_{1A}-molecules as a result of disulfide bond disruption. In a similar manner complement fixation that does not require covalent linkage has been shown to be less efficient in γ_2-globulin molecules that undergo disulfide bond reduction (55). Gamma$_{1A}$-complexes with β-lipoprotein and α_1-glycoproteins have also been reported (56, 57).

[9] One free-SH group is available in γ_2-globulin.

The fact that the serum level of γ_{1A} remained unchanged after penicillin therapy does not bear upon the loss of binding by the γ_{1A}, since the quantitation of the immunoglobulin depends only upon preservation of its antigenicity. The mild conditions of reduction employed in these studies would not be expected to destroy antigenicity.

Gamma$_{1A}$ appears to have a great tendency to associate both with other serum proteins and with itself so that polymeric forms exist and sedimentation values from 7 to 13 S are common (58).

The structural basis for the protein association is unknown. The extensive structural studies as applied to γ_2-globulin have not as yet been utilized in the study of γ_{1A} because of technical difficulties. However, the response of γ_{1A} to enzymatic digestion and disulfide bond reduction, its behavior on Sephadex columns, and the fact that its small polypeptide chain is antigenically very closely related to the light polypeptide chain of γ_2- and γ_{1M}-globulins suggest that the chain arrangement is similar to that of γ_2-globulin (59, 60). Gamma$_2$-myeloma does not show the same ability to interact with other proteins on a nonimmunological basis. The differences in primary structure of the polypeptide chains of the γ_{1A}-molecule, as opposed to the other immunoglobulins, and as manifested in differing electrophoretic mobility, antigenicity, and antibody activity, thus find further expression in this example of protein interaction.

The nature of the phenomenon observed in this instance may then be postulated as follows: Gamma$_{1A}$, because of an unexplained inherent tendency to bind with other protein molecules, combined with AHG to inhibit its activity. This suppression was reversible *in vivo* and *in vitro* by disulfide bond reduction resulting in either dissolution of covalent bonds between the myeloma and AHG or conformational changes of γ_{1A}, upon which the protein interaction was dependent. The latter interpretation is favored.

Whether the interaction of the γ_{1A} with other proteins is dependent upon its paraprotein characteristics or whether normal γ_{1A} present in equivalent amounts would demonstrate similar activity cannot be determined from these studies. Except in myeloma, serum levels as high as were present in the patient have not been observed in our experience.

The present studies would further indicate that in this particular instance the AHG inhibitor was not enzymatic in nature, a possibility that has been discussed by others (1–3, 30).

Summary

The plasma of a patient with γ_{1A}-myeloma demonstrated a defect in the first stage of clotting, i.e., an inhibitor against antihemophiliac globulin (VIII). The inhibitor differed in several respects from the usual circulating anticoagulant against Factor VIII. Its action was reversed by penicillamine *in vitro* and by penicillin *in vivo*. The γ_{1A}-myeloma appeared to be responsible for the inhibition, as evidenced by the loss of the inhibitory action when the myeloma protein was removed with specific anti-γ_{1A}-antiserum, the loss being directly proportional to the amount of antiserum used. The inhibitor, isolated by both starch block and continuous flow electrophoresis, was localized in those fractions highly enriched in γ_{1A}-protein. The inhibitor was reversed by disulfide reducing agents, which are known to interfere with γ_{1A}-activity. It was postulated that the disulfide bond reduction disrupted the interaction between the myeloma and antihemophiliac globulin proteins.

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