Disc Electrophoresis Study of Serum Proteins from Patients with Multiple Myeloma and Macroglobulinemia

By Salvador B. Zingale, Carlos A. Mattioli, Hans D. Bohner, and Maria P. Bueno

Analyses of the abnormal serum proteins of patients with plasma cell myeloma have shown them usually to consist of single protein peaks, when studied by moving boundary or paper electrophoresis.\textsuperscript{1} Recent studies performed by starch gel electrophoresis have shown that myeloma globulins may be very heterogeneous.\textsuperscript{2,4} Ultracentrifugal analysis also has shown some heterogeneity of certain myeloma proteins.\textsuperscript{5-10} In spite of apparent homogeneity in the classical electrophoretical methods, several components with a sedimentation rate between 7S and 19S have been described—"atypical macroglobulins."\textsuperscript{11} Recently, immunoelectrophoretic and ultracentrifugal studies have related the heterogeneous myeloma components to the $\beta_{2\lambda}$ globulins.\textsuperscript{9,12,13}

Globulins with a sedimentation rate between 7S and 19S have been described in serum of patients with rheumatoid arthritis and some other conditions.\textsuperscript{14} A detailed study from the standpoint of association and dissociation demonstrated that the heterogeneous components were complexes of 7S gamma globulin.\textsuperscript{14}

On paper electrophoresis macroglobulinemic sera yield patterns indistinguishable from those found in multiple myeloma. A diagnosis of macroglobulinemia can not be done without analyzing the serum in the ultracentrifuge or by immunologic procedures. Recently, it has been shown that the starch gel electrophoresis technic can be very valuable in the diagnosis of this condition.\textsuperscript{2,6,15}

A new electrophoretic method with some of the properties of the starch gel technic has been recently described by Ornstein and Davis: "disc electrophoresis."\textsuperscript{16}

In previous work, it was found that an early version of the disc electrophoresis method is a procedure of high resolution, simple to perform and easily reproducible.\textsuperscript{17-19} With the same original technic, excellent results have been obtained by others.\textsuperscript{20} Recently, Ornstein and Davis have reported a new version of the disc electrophoresis technic which has remarkable advantages over the original procedure.\textsuperscript{16}

We thought it useful to apply this technic to the study of the abnormal...
proteins from cases of multiple myeloma and macroglobulinemia with the following objectives in mind: (1) to determine whether this method would show the myeloma globulin heterogeneity reported with the starch gel technic; (2) to determine the applicability of disc electrophoresis to the study of the dissociation characteristics of the heterogeneous components; (3) to determine the value of disc electrophoresis in the differential diagnosis of myeloma and macroglobulinemia sera.

MATERIALS AND METHODS

Selection of patients. Thirty-one patients with multiple myeloma and seven with macroglobulinemia were included in this study. Selection of cases was made upon definite diagnosis made by clinical, histologic, and serum or urine evidences of multiple myeloma. In the macroglobulinemia cases, the diagnosis was confirmed by immuno-electrophoresis and analytical ultracentrifugation performed by methods described below.

In most of the cases blood was obtained by one of the authors. Sera were kept at 4°C in sterile conditions until the tests were performed. Five of the myeloma sera had been stored at -20°C for periods of up to 8 months.

Electrophoresis. Disc electrophoresis was performed by the method of Ornstein and Davis by the two following procedures: Method 1. This procedure was accomplished according to an earlier version of the technic as described in detail previously. In summary, the polyacrylamide gel was prepared by mixing equal amounts of the four following stock solutions: (a) 30 per cent acrylamide* and 0.8 per cent of N,N'-methylene-bis-acrylamide* in distilled water; (b) 1 ml. per cent beta-dimethylaminopropionitrile* and 0.8 ml. per cent triethanolamine in distilled water; (c) 0.03 per cent potassium ferricyanide in distilled water; (d) 2.8 per cent ammonium persulfate in distilled water. Glass cylinders, 100 mm. in length and 5 mm. inner diameter, were carefully topped at one end and placed in a vertical holder. The cylinders were filled to within 20 mm. of the top with the gel catalyzed solution. Water was then carefully overlaid to produce a flat surface. Once the polymerization was completed, the water was poured off and the tubes were inserted in a plastic container by means of a rubber grommet sealed to the container. This upper reservoir was supported by a metallic ring attached to a ring stand that allowed lowering until the bottom of the tubes were immersed in the buffer solution of the lower reservoir. Any bubble formed in the bottom of the tube was removed. The whole system was filled with the buffer solution and on filling the upper container air bubbles present in the tube above the gel were removed. Five μL. of serum were applied in a layer on the upper surface of the gel in the interphase buffer-gel. Runs were made at 7 ma. per tube during 1 hour and 15 minutes. The buffer employed was sodium borate 0.016 m, pH 9.2. The gels were removed from the glass cylinders by rimming with a fine needle and stained during 40 minutes with a 0.8 per cent Amidoschwarz B 10 solution in 7 per cent acetic acid. De-staining was performed electrophoretically by placing the gel in an electrical field. The gels were stored in tubes with 7 per cent acetic acid. Method 2. This technic was accomplished according to the latest version of the disc electrophoresis method as reported in detail by Ornstein and Davis. This procedure was carried out exactly as described by the authors.

Disc electrophoresis is capable of separating the proteins of normal human serum into over 20 fractions. The protein zones were identified with normal serum proteins previously isolated by starch block electrophoresis as reported in previous work. An investigation of 88 normal human sera indicated that, depending on the haptoglobin type, three basic patterns can be discerned by this technic.

No effort was made to quantitate the patterns; for the purpose of this work careful visual inspection was considered enough for the interpretation of the results.

*Obtained through the courtesy of Cyanamid Co.
Table 1.—Sera from Myeloma Patients with One Abnormal Band on Disc Electrophoresis—Comparison of Results Obtained by Paper and Immunoelectrophoresis

<table>
<thead>
<tr>
<th>CASE</th>
<th>No.</th>
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<th>2</th>
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<td>DISC</td>
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</tbody>
</table>

All data refer to the abnormal globulin fraction.

*Abnormal components with partial penetration into the polyacrylamide gel.

**Abnormal globulin did not penetrate the gel.

***Sera without abnormalities on paper and disc electrophoresis. Analysis of the patient’s urine revealed the abnormal globulin.

****Immunoelectrophoretic pattern of the “micromolecular” type.

Some of the sera were analyzed by Method 1 in the following buffers: sodium barbiturate 0.05 M, pH 8.6; phosphate 0.001 M, pH 7.5; and phosphate 0.01 M, pH 7.2.

Filter paper electrophoresis was performed as reported in previous communications. Total protein determinations were done by the Biuret technic.

Immunoelectrophoresis. This technic was performed by the Scheidegger micromethod as modified by Heremans. Barbiturate-HCl buffer of pH 8.6 and ionic strength 0.025 was used. The agar gel contained 1.5 per cent of Special Agar Noble (Difco).

Three polyvalent antisera against human serum and two anti-gamma globulin sera were used in these studies. The antisera were made in rabbits as described by Heremans. Specific antisera anti-beta-2A globulin commercially obtained from Behringwerke A.G. was kindly furnished by Dr. Alois Bachmann of the National Academy of Medicine.

Ultracentrifugation. Ultracentrifugal analyses were carried out in a Spinco Model E analytical ultracentrifuge. All the studies were made using the single sector cell. Most of the runs were performed at 52,640 rpm.; in other instances 59,780 was employed. The sera were analyzed at a protein concentration of 0.8 to 1.2 Gm. per cent diluted in 0.15 M NaCl. Sedimentation constants were not corrected for concentration dependance. Determination of sedimentation constants and concentration were performed as described by Elias.

Isolation of pathologic components. In six myeloma sera, the abnormal protein was isolated in starch according to the Kunkel’s technic as reported elsewhere.

Dissociation experiments. Whole serum or isolated fractions were treated with one of the following reagents: urea 6 M, ethyl mercaptan 0.1 M, cysteine hydrochloride 0.1 M, acetate
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Table 2.—Sera from Myeloma Patients with More Than One Abnormal Band on Disc Electrophoresis—Results of Ultracentrifugal, Paper and Immunoelectrophoretic Analyses

<table>
<thead>
<tr>
<th>CASE</th>
<th>No.</th>
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<td>3.38</td>
<td>(\alpha_1)</td>
<td>(\beta_{2A})</td>
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</table>

All data refer to abnormal globulin fractions.

buffer 0.1 M, pH 4.1. Proteins treated by these agents were submitted to disc electrophoresis. Likewise they were analyzed in the ultracentrifuge.

RESULTS

In tables 1 and 2 are shown the results of disc, paper and immunoelectrophoresis in patients with multiple myeloma.

On paper electrophoresis every serum studied but one (Case 22) was found to have a typical myeloma peak. Serum 22 belongs to a patient with a classical myeloma in which the pathologic globulin was detected in the electrophoretic study of the urine.

By means of disc electrophoresis two major groups of myeloma sera could be easily distinguished: (A) sera with only one abnormal component; (B) sera with several abnormal components.

Group A. Of the 31 myeloma sera, 24 were found to have only one abnormal component. The pathologic globulin had a mobility that differed from serum to serum and they were found to migrate at various rates from the slower gamma globulin to the region of the fastest beta globulin (table 1 and fig. 1).

In some sera the abnormal band was very sharp, in other instances they were diffuse indicating some degree of heterogeneity. In one of the sera (Case 21), the abnormal protein did not penetrate into the gel. This serum was
Fig. 1.—Disc electrophoresis of homogeneous myeloma proteins. A. Disc electrophoresis Method 1. B. Disc electrophoresis Method 2. (In all illustrations showing electrophoretic patterns, the anode is toward the left and the cathode is toward the right). See explanation in text.
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Fig. 2.—Immunoelectrophoresis patterns from several patients with myeloma. In Case 25, A corresponds to the isolated fraction; B, whole serum. Note in B the arc close to the cup and the identity reaction with the albumin arc.

examined by disc Method 1 in a variety of borate, phosphate and barbiturate buffers. In none was a clear penetration obtained. The sedimentation constant of this particular globulin was 6.3S.

The immunoelectrophoretic studies of this group of sera revealed that all cases but two were of the gamma type (table 1).

Ten sera analyzed in the ultracentrifuge showed only one abnormal peak in the area of the 7S globulins.

Group B. Multiple abnormal components were observed in seven sera. In four cases four were seen, and in three sera two were found (fig. 3). In all cases the abnormal peaks showed as a group of slow, very homogeneous bands preceded by a single band always diffuse and highly concentrated (fig. 3). The former bands frequently appeared superimposed on the zone of haptoglobins. In some instances, particularly in sera of the 2–1 and 2–2 haptoglobin type, it was difficult to differentiate the abnormal components from the haptoglobins. However, specific stain for haptoglobins allowed a clear distinction of the abnormal bands.

Although there were some changes in mobility, the distribution and number of the multiple bands persisted with similar characteristics when phosphate and barbiturate buffers were used.

In five sera the abnormal globulin was isolated by starch block electrophoresis. The isolated proteins resolved into similar components on gel than could be demonstrated in the whole serum.

A striking correlation between the heterogeneity observed in the gel and heterogeneity in the ultracentrifuge was found. As can be seen in table 2, in every case in which several abnormal bands appeared in the gel, the same number of peaks were observed in the ultracentrifugal pattern. A comparison of the relative concentration of the abnormal bands observed in the gel with the abnormal peaks detected by the ultracentrifuge revealed that the fast components in the gel corresponded to the slower peaks in the ultracentrifuge.
Thus, it could be established that the fast, diffuse band observed in the gel was the 7S component of the abnormal protein. The slower bands in the gel were related to a group of components with a sedimentation rate between 7S and 19S. The uncorrected sedimentation rate and relative concentration of the latter components can be seen in tables 2 and 3. In three cases, those globulins represented more than 40 per cent of the total globulins.

The immunoelectrophoresis patterns revealed a \( \beta_{2A} \) antigenic structure in all the heterogeneous sera. Four cases showed an abnormal accessory arc in the beta region in close vicinity of the starting cup. In one case, the abnormal arc fused completely with the cathodic end of the albumin arc (fig. 2).

**Dissociation experiments.** Sera which had been clearly heterogeneous in both disc electrophoresis and ultracentrifugation were selected for these experiments (table 3). The heterogeneous pattern remained unchanged when ultracentrifugation was performed in the presence of acetate buffer, pH 4.1. Occasionally, the faster peak seemed to disappear almost completely from the effect of the acid buffer. It should be emphasized that these experiments were difficult to evaluate because in most instances certain protein denaturation occurred. The latter circumstance made it very difficult to distinguish the abnormal peaks from similar ones produced by the acid buffer.

None of the abnormal components dissociated from the effect of urea 6 M. Some of the peaks were clearly dissociated by sulfhydryl liberating compounds. The faster components of the heterogeneous sera were readily dissociated in the presence of ethyl mercaptan 0.1 M. When four peaks were present, the reducing agent led to a complete or almost complete disappearance of the two faster components. A simultaneous increase in the area of the 7S peak was also detected in three sera (table 3). The same cases analyzed by disc
Table 3.—Effect of Sulfhydryl-liberating Compounds on Electrophoretic and Ultracentrifugal Behavior of Heterogenous Myeloma Globulins

<table>
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<th>CASE No.</th>
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<th>Sera with Sulfhydryl reagent</th>
<th>Sera without Sulfhydryl reagent</th>
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<tbody>
<tr>
<td></td>
<td>Relative concentration of bands</td>
<td>Relative concentration of bands</td>
<td>No. of peaks %20 (%)</td>
<td>No. of peaks %20 (%)</td>
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<td>6.8 17.0</td>
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<td>6.8 17.0</td>
<td>6.3 17.0</td>
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</table>

All data refer to abnormal globulin fractions.
 Relative concentration determined by visual estimation.
 Relative per cent concentration of abnormal peaks.
electrophoresis revealed a marked decrease in the concentration of the two slower bands (fig. 3). When only one extra component was present it disappeared completely from the effect of ethyl mercaptan (table 3 and fig. 4. Cases 28 and 31). Cysteine hydrochloride 0.1 M had a similar effect. However, in three sera it did not produce as clear an effect as the one obtained with ethyl mercaptan.

Macroglobulinemia cases. Sera from seven patients with macroglobulinemia were examined with the disc electrophoresis method. In all cases a sharp band at the origin of the run in a zone of about 1.5 mm. thickness was observed (fig. 5). A marked increase of the 7S peak was seen in the ultracentrifugal pattern of one case. This abnormality was also clearly demonstrated by the disc electrophoresis technic (fig. 5, Case 2). When the sera were previously treated with ethyl mercaptan 0.1 M, the macroglobulins dissociated as demonstrated by the almost complete absence of band at the origin and the apparition of a new component in the fast zone of the gamma globulin (fig. 5).

Discussion

Since a parallel study with starch gel electrophoresis was not carried out, no comparison is possible between the utility of starch gel and disc electrophoresis in studies of myeloma proteins. The disc electrophoresis method revealed a clear heterogeneity in 22 per cent of the myeloma sera. This incidence, as well as the pattern of heterogeneity observed, seems to be com-

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Fig. 4.—Ultracentrifugal patterns from three heterogeneous myeloma sera. A. Serum without sulfhydryl reagent. B. Serum treated with ethyl mercaptan 0.1 M. Photographs taken at 94 minutes. Sedimentation proceeds from right to left. Note in B the absence of the faster peaks.

parable with the observations reported by several authors. However, it has been recently reported that 70 per cent of the myeloma globulins are heterogeneous in starch gel electrophoresis when studied at low protein concentration in glycine buffer. This heterogeneity was found to reflect differences in net electrical charge and molecular size; thus two types of heterogeneity (electrophoretic and polymer type) could be demonstrated with starch gel electrophoresis.

The disc electrophoresis technic seems to reveal only the polymer type of heterogeneity. The multiple components reflected differences in molecular size rather than in charge. Thus, the heterogeneous globulins resolved into the same number of components on gel that could be demonstrated in the ultracentrifuge. Furthermore, they revealed similar heterogeneity when studied in disc electrophoresis in a variety of buffers. It remains to be determined
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Fig. 5.—Disc electrophoresis of serum from patients with macroglobulinemia. A. Note the sharp band at the origin of the runs. B. Note the appearance of a band in the gamma 7S zone from the effect of sulfhydryl-liberating compounds. See explanation in text.

whether the starch gel electrophoretic type of heterogeneity of the myeloma proteins would be revealed by the disc electrophoresis technic if the conditions specified by Fahey were adhered to.

The lack of dissociation of the heterogeneous components in the presence of acid buffers and urea 6 M indicates that these globulins do not represent the type of complexes observed in rheumatoid arthritis sera by Kunkel et al.

The easy dissociation of the heavier components from the effect of sulfhydryl-liberating compounds suggests a role of disulfide bonds in their structure. This behavior has been interpreted as indication of polymer formation of 7S molecular units through binding at sites containing SH-groups. However, the possibility that the heterogeneous globulins represent other types of protein complexes must be considered.

Heremans has demonstrated that the β2A globulin has a marked complexing tendency with albumin. Moreover, the complex β2A globulin albumin can be dissociated by either cysteine or mercaptoethanol. These complexes are revealed by immunoelectrophoresis as one or several arcs located in the beta region.

In the present study an abnormal arc with the above-mentioned characteristics was observed in four heterogeneous sera. In one case it had an identity reaction with the arc of the albumin. Furthermore, all the heterogeneous sera were of the β2A type and some of the abnormal components were readily dissociated by sulfhydryl-liberating compounds. These observations suggest that at least some of the heterogeneous components might not be the expression of polymer formation of 7S molecular units. The final answer to this problem must await more studies of protein dissociation and physicochemical characterization of the isolated components.

Seven cases of macroglobulinemia were examined by the disc electrophoresis technic. This method made it possible to distinguish myeloma globulins from macroglobulins. However, in two cases of myeloma the appearance of the
abnormal band happened to be similar to the one usually seen in macroglobulinemia. Definite distinction was attained when the serum was treated with either ethyl mercaptan 0.1 M or cysteine hydrochloride 0.1 M. From the effect of the reducing agents the macroglobulins dissociated in subunits that penetrated into the gel. In all cases, the disc electrophoresis method revealed some degree of penetration of the abnormal globulin though a certain amount of protein remained at the origin. This latter finding contrasts with the starch gel electrophoresis studies which have shown that abnormal macroglobulins do not penetrate into starch gels. The different behavior of the macroglobulins in the polyacrylamide gel may be explained on the basis of the almost complete absence of endosmotic flow of the gel.

CONCLUSION

The disc electrophoresis technic provides a procedure of high resolution and is simple to perform. The use of polyacrylamide gel as a supporting medium also has the advantage of being completely transparent, allowing a detailed characterization of myeloma protein abnormalities not revealed by filter paper electrophoresis. The technic can be used for protein dissociation experiments with urea and sulfhydryl liberating compounds. In its present form, however, it does not seem to demonstrate all the myeloma protein abnormalities revealed by starch gel electrophoresis.

Disc electrophoresis is a valuable procedure for the diagnosis of macroglobulinemia.

SUMMARY

Sera from 31 patients with plasma cell myeloma and seven with macroglobulinemia were analyzed by the disc electrophoresis method (Ornstein and David).

Of the 31 myeloma cases, 24 were found to have a single component. In the remaining cases, in spite of apparent homogeneity in paper electrophoresis, several abnormal components were seen.

Ultracentrifugal studies revealed that the heterogeneous myeloma components are a group of globulins with a sedimentation rate between 7S and 19S. Some of the latter components could be easily dissociated by sulfhydryl liberating compounds.

Comparison of the results with those obtained by paper and immunoelectrophoresis demonstrated that all the heterogeneous proteins were fast globulins of the $\beta_2$ type.

The possible significance of the heterogeneous myeloma proteins is discussed.

SUMMARIO IN INTERLINGUA

Seros ab 31 patientes con myeloma a cellulas de plasma e ab septe con macroglobulinemia esseva analysate per le metodo del electrophorese a disco (Ornstsein e David).

Inter le 31 casos de myeloma, il esseva trovate que 24 habeva un sol componente anormal. In se remanente casos—in despecto del apparente homo-
geneitante in electrophorese a papiro—plure componentes anormal esseva distinguible.

Studios de ultracentrifugation revelava que le heterogenee componentes in myeloma es un gruppo de globulinas con un sedimentation de inter 7S e 19S. Plures de iste ultime componentes esseva facile a dissociar per medio de compositos liberatori sulfhydrylic.

Comparation del resultados con illos obtenite per electrophorese a papiro e immunoelectrophorese demonstrava que omne le heterogenee proteinas esseva rapide globuiinas del typo beta $\alpha$.

Le signification possibile del heterogenee proteinas in myeloma es discutite.

ACKNOWLEDGMENT

One of the authors (S. B. Z.) wants to express his gratitude to Drs. Stanley L. Lee, Morris Wasserman and Baruch J. Davis for their assistance and advice in learning the disc electrophoresis technic. The authors are also very grateful to Drs. Rosa Pirosky, Gregorio Bonchil, Alois Bachmann, Manuel Arce, Alfredo Pavlosky and Fritz Schajowitz for their kind cooperation in permitting us the use of their patients for this study. We acknowledge the valuable cooperation of Dr. Arnoldo Marino for the preparation of the illustrative material presented in this work.

REFERENCES


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