IMMUNOCHEMICAL STUDIES OF TWENTY MOUSE MYELOMA PROTEINS: EVIDENCE FOR TWO GROUPS OF PROTEINS SIMILAR TO GAMMA AND BETA-2A GLOBULINS IN MAN

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The myeloma globulins formed in malignant plasma cells have particular importance as accessible and relatively discrete products of plasma cell metabolism which closely resemble normal immune globulins. Serum myeloma proteins obtained from patients with multiple myeloma have been well characterized and immunochemical differences recognized which have led to the division of the myeloma proteins into two major groups, usually designated gamma (γ) type and beta (β2A) type myeloma proteins, on the basis of distinctive antigenic properties (1–4). Identification of the two types of myeloma proteins facilitated recognition of the normal β2A-globulins as one of the human serum components formed in plasma cells. Since β2A-globulins had been identified only in human serum, it was not certain whether these proteins were unique to man or whether they occurred in other species as well. The finding of similar globulins in the mouse in the course of the present studies, therefore, was of particular interest.

The recent discovery of transplantable plasma cell tumors (5–9) and leukemias (10, 11) in mice with associated serum myeloma proteins and urinary Bence Jones proteins makes possible a new approach to the study in experimental animals of normal and malignant plasma cells and their metabolic products.

The present studies were undertaken to determine the immunochemical characteristics of the myeloma proteins associated with 20 transplantable plasma cell tumors from two strains of mice and to relate them to normal components of mouse serum. In the course of this work it became apparent that mouse myeloma proteins, like human myeloma proteins, could be divided into two groups having distinctive antigenic and physicochemical properties. One group of myeloma globulins was closely related to the normal gamma globulins of mouse serum but the other group was related to a separate normal serum component of beta mobility.

Methods

Sera were obtained from mice bearing transplantable mouse plasma cell tumors which had been maintained by subcutaneous implantations of tumor fragments.[1]

Four of the tumors (5563, 5647, SPC-1, DPC-1) originated in C_3H mice and the remainder originated in BALB/c mice. The origin, biologic behavior, and histological characteristics of these tumors have been or will be reported elsewhere (5–9, 12). Myeloma proteins or sera from mice bearing plasma cell tumors are designated by the name of the tumor, *i.e.*, 5563 myeloma proteins are associated with the 5563 plasma cell tumor. Normal mouse serum was obtained from C_3H/He or BALB/c mice by cardiac puncture and the sera from each strain was pooled separately, frozen, and stored at −20°C until used for analysis.

Immunoelectrophoresis was performed on microscope slides by Wieme's modification of the technique of Scheidegger (13, 14) or on 3¼ X 4 inch photographic plates covered with 18 ml of 1 per cent ionogar in 0.045 λ veronal buffer. A current of 1.5 ma/cm was employed for 2 hours at room temperature in a box with high humidity to separate the protein components. The modified immunoelectrophoretic technique of Osserman (15) was employed to relate the myeloma proteins to components of normal serum. Ouchterlony double diffusion analysis in agar plates was carried out as described previously (16).

Antisera were produced by immunizing rabbits with normal mouse serum (4 rabbits), normal mouse gamma globulin (4 rabbits), 5563 myeloma protein (4 rabbits), 5647 myeloma protein (6 rabbits), and MPC-2 myeloma protein (2 rabbits).[2]

1 ml of serum or 2 to 5 mg of purified protein in 1 ml saline was emulsified in 1 ml of complete Freund's adjuvant and injected into two intramuscular sites. This injection was repeated in 2 weeks and the rabbits were bled approximately 3 to 5 weeks later. Some animals also received a course of intravenous injections of alum-precipitated protein and were bled several times throughout the following week.

Purified 5563 and MPC-11 myeloma proteins were prepared from serum by anion-exchange cellulose chromatography employing a 0.015 M to 0.05 M phosphate gradient (16). MPC-1, MPC-2, and 5647 myeloma proteins were prepared by a two step fractionation process employing first zone electrophoresis of serum on polyvinyl chloride particle (geon 426, B. F. Goodrich Chemical Co., Cleveland) blocks by a modification of the procedure of Müller-Eberhard and Kunkel (16, 17) to obtain the electrophoretic fraction containing the myeloma globulin. This was subfractionated by means of diethylaminoethyl (DEAE) cellulose chromatography employing a gradient elution (16) from 0.04 M to 0.30 M phosphate pH 8 buffer. Each purified protein was characterized by starch gel electrophoresis, immunoelectrophoresis, and ultracentrifugation and found to contain at least 95 per cent myeloma protein, and in most preparations no contaminant proteins were detected. Normal gamma globulin was obtained by eluting serial sections in the gamma globulin region after block electrophoresis of normal or hyperimmunized mouse sera. Each fraction was tested by the Ouchterlony technique with rabbit antisera prepared against mouse serum. Only those electrophoretic fractions showing a single (γ) precipitin line were pooled for subsequent testing and use as gamma globulin.

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[1] Tumors 5563, 5647, MPC-1, 2, 11, 14, and 15 were maintained in this laboratory by serial transplantation from tumors generously made available by R. Merwin and M. Potter. Sera from mice bearing the SPC-1 and DPC-1 tumors were made available by M. Potter and sera from mice bearing tumors MPC-3, 4, 5, 6, 7, 8, 16, 17, 19, 20, and 21 were made available by R. Merwin.

[2] Eight of these antisera were prepared in association with Dr. Brigitte A. Askonas (National Institute for Medical Research, London, England), who also kindly made available two of the antisera against 5563 myeloma proteins.

RESULTS

Immunoelectrophoresis.—Immunoelectrophoresis of normal mouse serum revealed multiple components among the normal serum proteins (Fig. 1). In addition to the normal serum components each myeloma serum showed a relatively dense precipitation arc indicating the presence of myeloma globulin when an appropriate antisera was used (Figs. 1 to 3). The myeloma globulins were found to have the electrophoretic mobility of γ-, β-, or α-globulins on immunoelectrophoresis in agreement with the electrophoretic mobility observed on paper or moving boundary electrophoresis (Fig. 4). The MPC-15 myeloma protein, however, migrated very little on immunoelectrophoresis since its

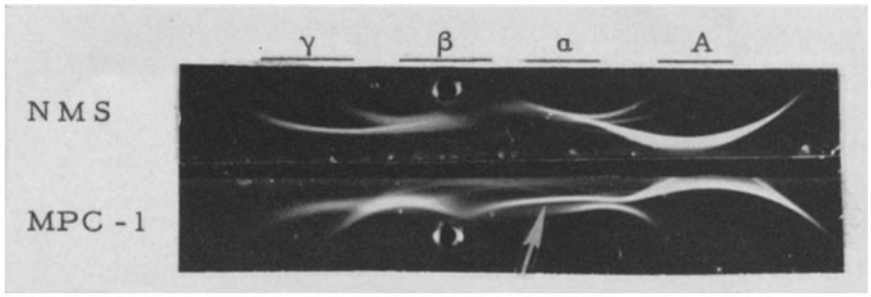

FIG. 1. Immunoelectrophoresis of normal mouse serum (NMS) and mouse myeloma sera (MPC-1). Electrophoresis was carried out in 0.045 ionic strength barbital buffer pH 8.6 and rabbit antisera prepared against normal mouse serum was allowed to diffuse from the central trough. The arrow indicates the precipitin arc due to the myeloma protein of alpha globulin electrophoretic mobility in MPC-1 serum.

euglobulin properties caused it to precipitate in the 0.045 ionic strength buffer used (Fig. 3).

The mouse myeloma proteins could be divided into two groups, designated gamma and beta types on the basis of differences in the predominant antigenic properties. This difference is shown in Fig. 2 where two gamma type myeloma globulins, 5563 and MPC-11, are shown to react strongly with antisera prepared against normal gamma globulin. A similar reaction is seen when antisera to gamma myeloma proteins is used. These same myeloma globulins, however, react poorly with antisera prepared against beta type myeloma proteins (Fig. 3). Conversely, in Fig. 3, two beta type myeloma proteins (5647 and MPC-1), are shown to react strongly with antisera prepared against beta type myeloma protein, but weakly with anti-gamma antisera. The remaining 16 myeloma proteins, (SPC-1, DPC-1, MPC-2, 3, 4, 5, 6, 7, 8, 14, 15, 16, 17, 19, 20, and 21) reacted well with antisera to beta type myeloma proteins but poorly with antisera to gamma globulins. The reaction to antisera prepared against MPC-2

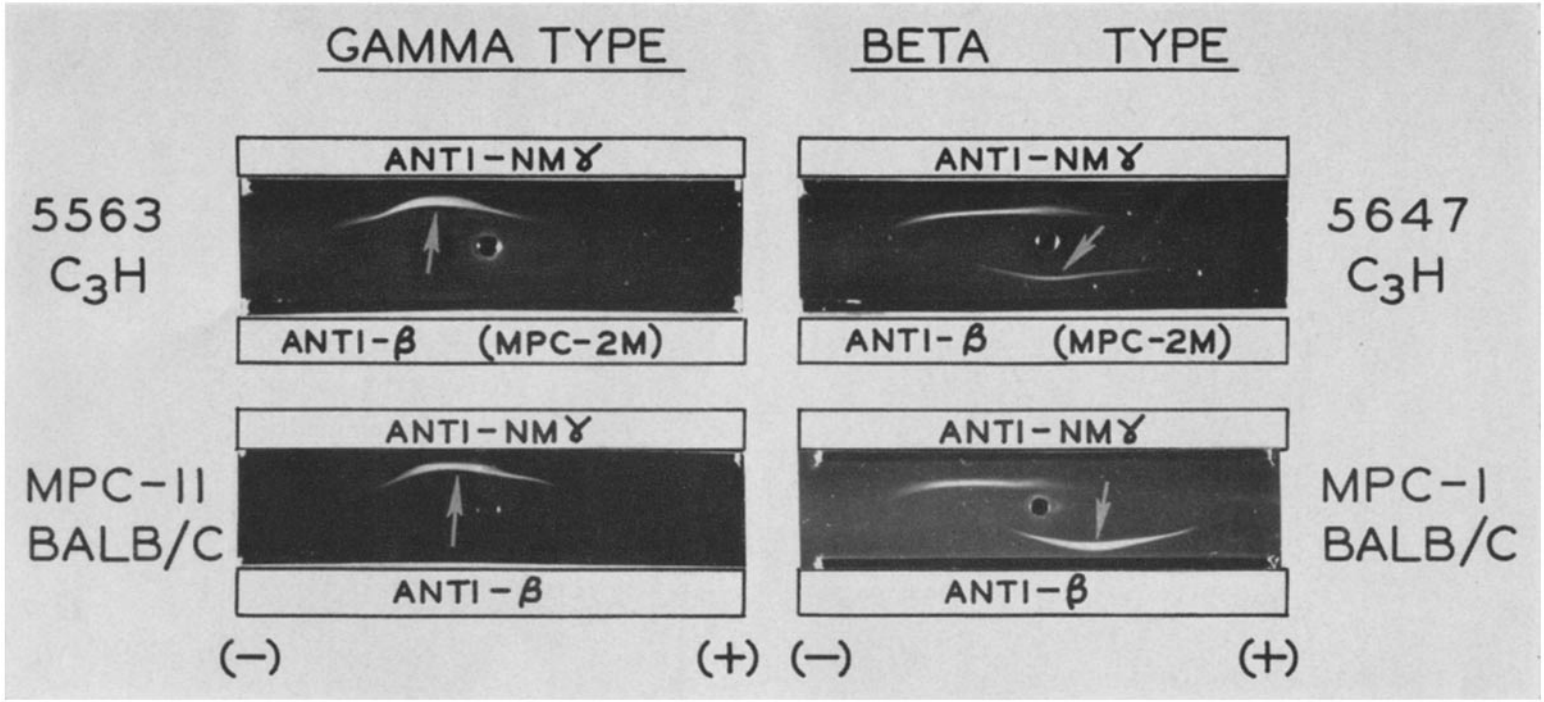

FIG. 2. Two immunoelectrophoretic types of mouse myeloma proteins were revealed by reaction with specific antisera. After agar electrophoresis of each myeloma serum, the upper trough was filled with rabbit antisera prepared against normal mouse gamma globulin (anti-NMγ) and the lower trough was filled with rabbit antisera prepared against a beta type myeloma protein (anti-β). Antisera *vs.* purified MPC-2 beta myeloma protein was employed in the studies shown above, but similar results were obtained when antisera *vs.* 5647 beta myeloma protein was used. Myeloma proteins which reacted strongly with antisera against gamma globulin and caused a localized bulge in the gamma globulin precipitin arc were classified as gamma type myeloma proteins (*i.e.*, 5563 and MPC-11). Myeloma proteins, however, which reacted strongly with antisera against beta myeloma proteins were classified as beta type myeloma proteins. In the 5647 and MPC-1 sera illustrated above the myeloma protein is indicated by the arrow. The normal gamma globulin component in these sera is seen as a long arc produced by reaction with anti-NMγ.

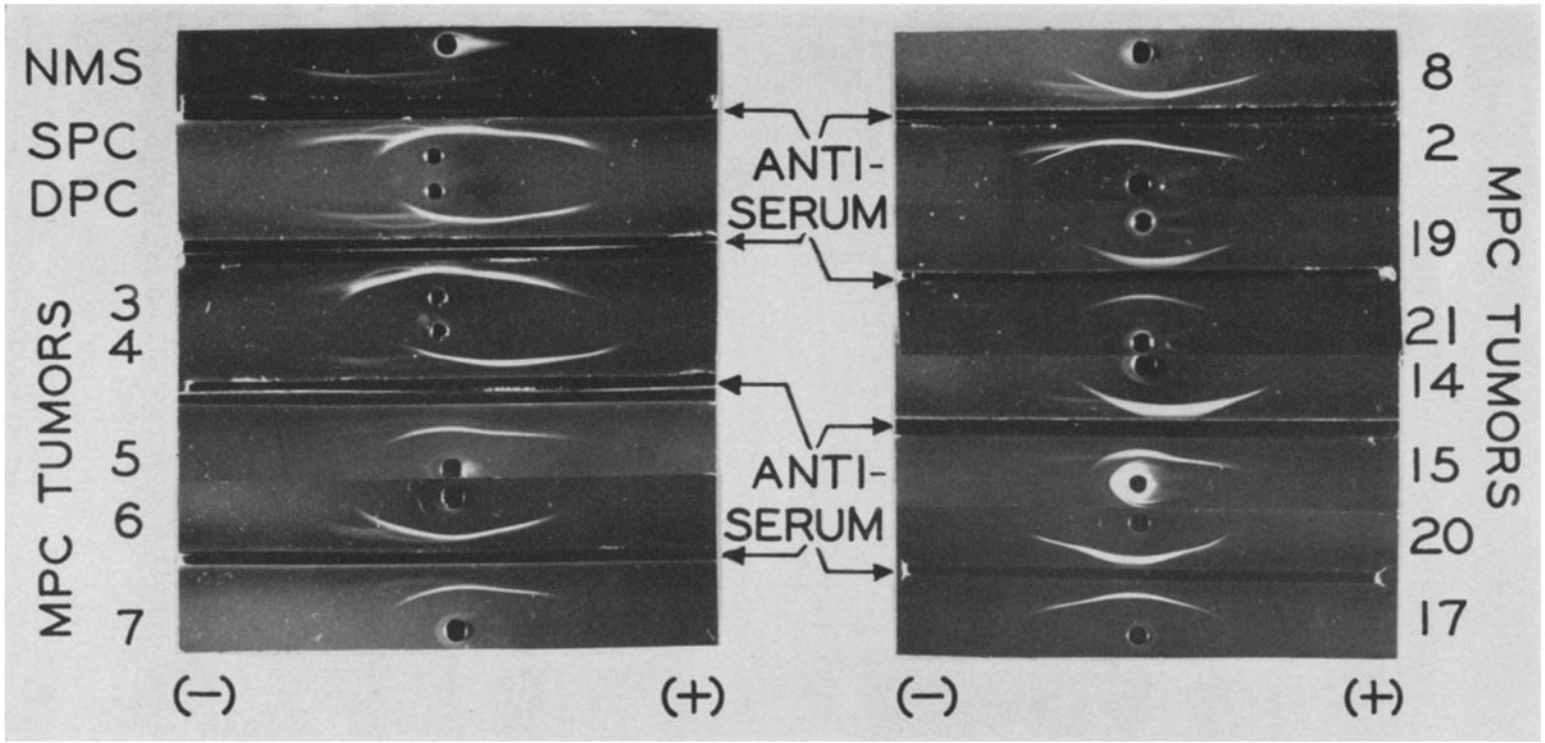

FIG. 3. Immunoelectrophoresis of 15 myeloma sera with beta type myeloma proteins. The SPC and DPC tumors developed in C_3H mice and the remaining tumors occurred in BALB/c mice. The rabbit antisera employed in this test was prepared against purified MPC-2 myeloma protein. In addition to the major precipitin arc indicating the myeloma protein, a contiguous cathodal precipitin band indicating the normal gamma globulin component was seen after prolonged reaction and was presumed to be due to the presence of antigenic determinants on the gamma globulins which are present as well on the beta type myeloma protein molecules (see Results, Fig. 7).

myeloma protein is shown in Figs. 2 and 3 and similar reactions were observed to antisera against the 5647 myeloma protein.

The capacity to develop plasma cell tumors producing gamma or beta myeloma protein was present in both C_3H and BALB/c strains of mice since myeloma proteins of the C_3H tumor 5563 and the BALB/c tumor MPC-11 were gamma type globulins, and beta type myeloma proteins were found with the C_3H tumors 5647, SPC-1, and DPC-1, and with most MPC lines of BALB/c tumors. Also, the fact that antisera prepared against myeloma globulins from both strains of mice distinguished between the two myeloma globulin groups is further evidence that strain differences were not responsible for the gamma and beta type myeloma globulin differences.

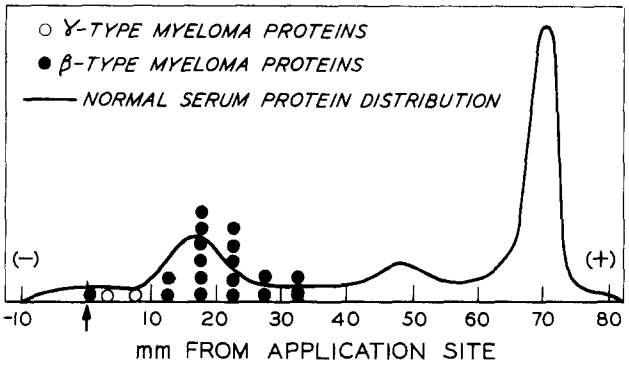

FIG. 4. Distribution of 20 myeloma proteins on zone electrophoresis in comparison with the normal mouse serum protein pattern. The location of the mid-portion of each myeloma protein peak is indicated by one circle. The mid-portion of the peak of albumin was at +70 mm, alpha-2 globulin at +48 mm, and beta globulin at + 17 mm.

Relation to Normal Serum Components.—The antigenic relationship of the myeloma proteins to normal serum components was shown by use of the immunoelectrophoretic modification introduced by Osserman (15). In Fig. 5, normal mouse serum was subjected to immunoelectrophoresis and from the trough on opposite sides of the electrophoresed serum were allowed to diffuse purified myeloma protein or antiserum to mouse serum proteins. When gamma type 5563 myeloma protein was allowed to diffuse from one trough and antisera from the other, the single precipitin line running the length of the troughs and representing the gamma myeloma protein was seen to react with the normal gamma globulin precipitin arc (Fig. 5).

Beta type myeloma proteins, however, were found to be closely related to a normal beta globulin component and not to gamma globulin, in experiments such as that illustrated in Fig. 5. In this test agar electrophoresis of normal mouse serum was carried out in the usual way and a mixture of antisera *versus*

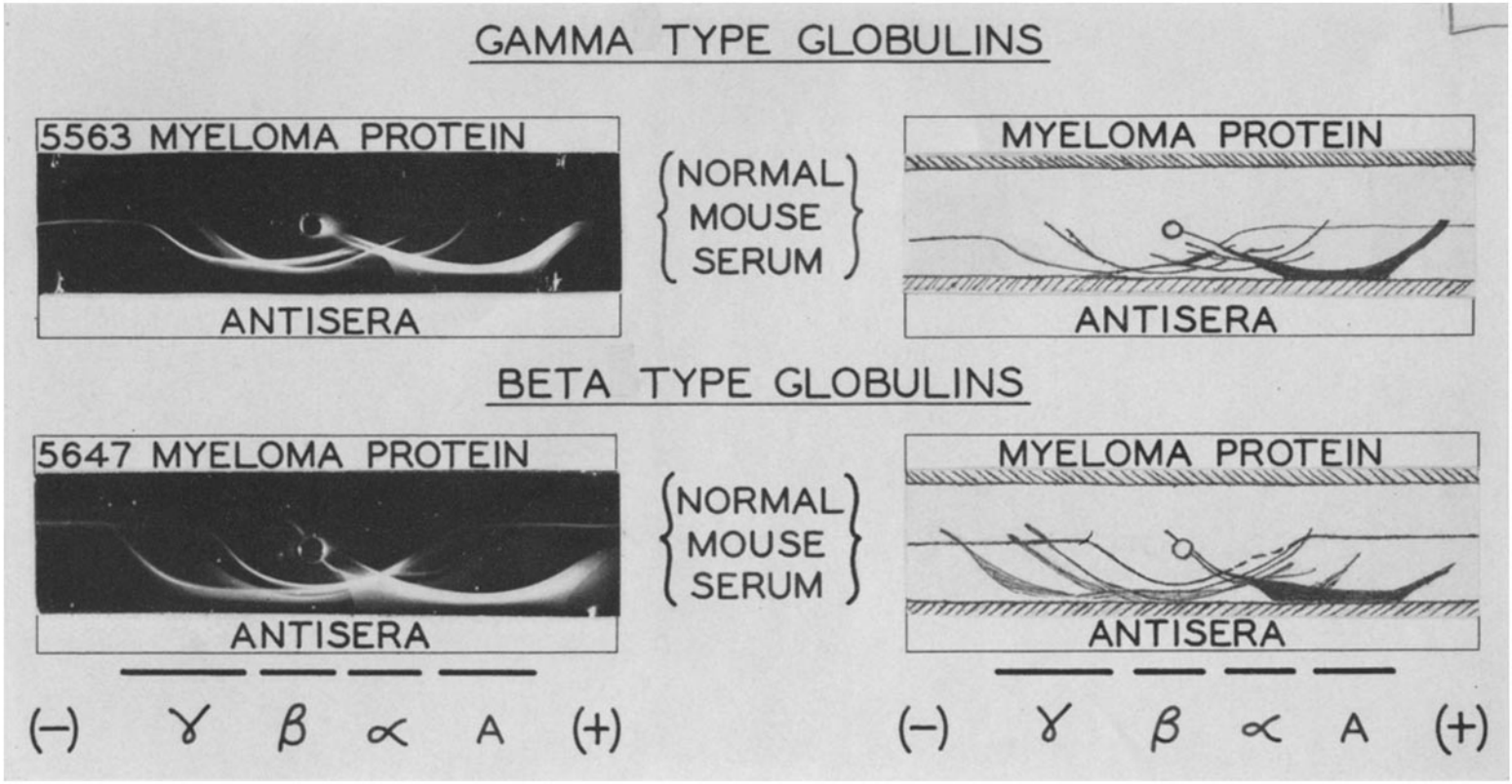

FIG. 5. Identification of normal serum components related to mouse myeloma globulins. In the upper figure, after electrophoresis in agar of normal C_3H/He mouse serum, purified gamma type myeloma protein was allowed to diffuse from the upper trough, and in the opposite (lower) trough was placed rabbit antisera prepared against normal mouse serum. In the lower figure beta type myeloma globulin was added to the upper trough after electrophoresis of normal BALB/c serum and a mixture of antisera to normal mouse serum and to beta type myeloma proteins was added to the lower trough to demonstrate the relationship of beta myeloma protein to a normal beta globulin component. Photographs were made after 4 days at 5°C.

normal mouse serum and antisera *versus* beta myeloma protein was added to one trough. To the opposite trough, however, was added purified myeloma protein. Under these conditions a long transverse precipitin band representing the myeloma protein formed midway between the two troughs. As is shown in Fig. 5, this horizontal band deviated toward the antibody trough in the beta region, joining the precipitin band formed by a normal serum component and indicating the presence in normal serum of a normal globulin related to the

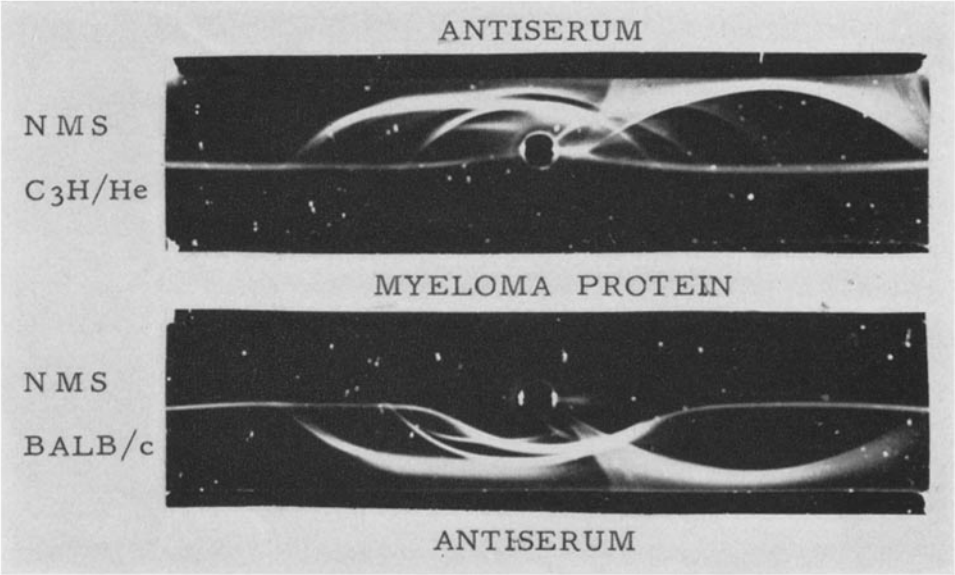

FIG. 6. Comparison of the normal beta globulin component in two strains of mice. Fresh sera obtained from BALB/c and C_3H/He mice were used. Purified MPC-1 myeloma protein (0.3 mg/ml) was allowed to diffuse from the center trough and the mixture of rabbit antisera to normal mouse serum, and to MPC-2 and 5647 myeloma proteins was added to the outer troughs.

beta myeloma protein. Myeloma proteins from different species (BALB/c and C_3H) and differing electrophoretic mobility (5647 and MPC-2 myeloma proteins migrate among the beta globulins and MPC-1 myeloma protein among the alpha globulins) reacted with the same beta component of normal serum (see Figs. 5 and 6). The gamma globulin precipitin arc was seen to pass through the beta myeloma precipitin band (Fig. 5), further emphasizing the antigenic distinction between the gamma and beta globulin components.

Strain differences in the normal beta component were detected. When beta type myeloma proteins were reacted with fresh C_3H and BALB/c serum after immunoelectrophoresis, shown in Fig. 6 for the MPC-1 globulin, the myeloma

protein reaction with the beta component in BALB/c serum was much more readily evident than with C_3H serum. The same differences were observed with purified MPC-2 and 5647 (Fig. 5) myeloma proteins and was not dependent upon the strain of origin of the myeloma protein or the type of antisera employed.

The beta type mouse myeloma proteins also showed a precipitin band that appeared to join with the albumin precipitin arc when antisera *versus* whole mouse serum was employed (Fig. 1). No reaction with albumin was observed, however, when antisera against beta type myeloma proteins (or gamma globulin) was used (Figs. 2, 3). A similar observation was noted by Heremans (4) with beta-2A myeloma proteins in man.

Comparison of Purified Myeloma Proteins.—Ouchterlony double diffusion agar plates were used for direct comparison of four representative γ- and β-type myeloma proteins. By placing γ- and β-type myeloma proteins in adjacent cups (Fig. 7 *D*, *E*) and using suitable antisera, the γ-myeloma proteins were shown to have antigenic sites that were not present on β-type globulins (spur formation for the γ-myeloma protein, Fig. 7 *B*). Also β-myeloma globulins were shown to have distinctive antigenic sites that were not present on the γ-myeloma globulins (spur formation in the precipitin bands for β-myeloma proteins in Fig. 7 *E*) or normal γ-globulins. This illustration of distinctive properties by direct comparison of purified γ- and β-myeloma proteins confirms the immunoelectrophoretic observation that these two types of proteins possessed distinctive antigenic determinants.

In addition to the specific antigenic determinants which permitted distinction between the γ- and β-classes of myeloma protein, these classes also were found to share common antigenic sites. A precipitin band, such as that illustrated in Fig. 7 *E* between the antisera and the γ- myeloma protein, which fused with but did not penetrate through the heavier precipitin band of the β-globulin, indicated that the γ-myeloma protein shared one or more antigenic determinants with the β-myeloma globulin. Similarly, in Fig. 7 *D* a precipitin band formed by the β-myeloma protein fused with the heavier γ-precipitin band, indicating that an antigenic determinant is shared by the γ- and β-myeloma proteins.

Proteins within the γ- or β-groups appeared to be closely related antigenically. Fusion of precipitin bands formed by two adjacent γ- myeloma proteins (Fig. 7 *A*) and by two adjacent β-myeloma proteins (Fig. 7 *C*) indicated that the proteins within each group shared antigenic determinants. Antigenic differences as well as antigenic similarities were also found when individual members of each group were compared further. The 5563 γ-myeloma protein, for example, is shown by the spur formation in Fig. 7 *B* to have antigenic determinants that are not shared by the MPC-11 γ-myeloma protein. Similar differences were observed when individual β-myeloma proteins were compared.

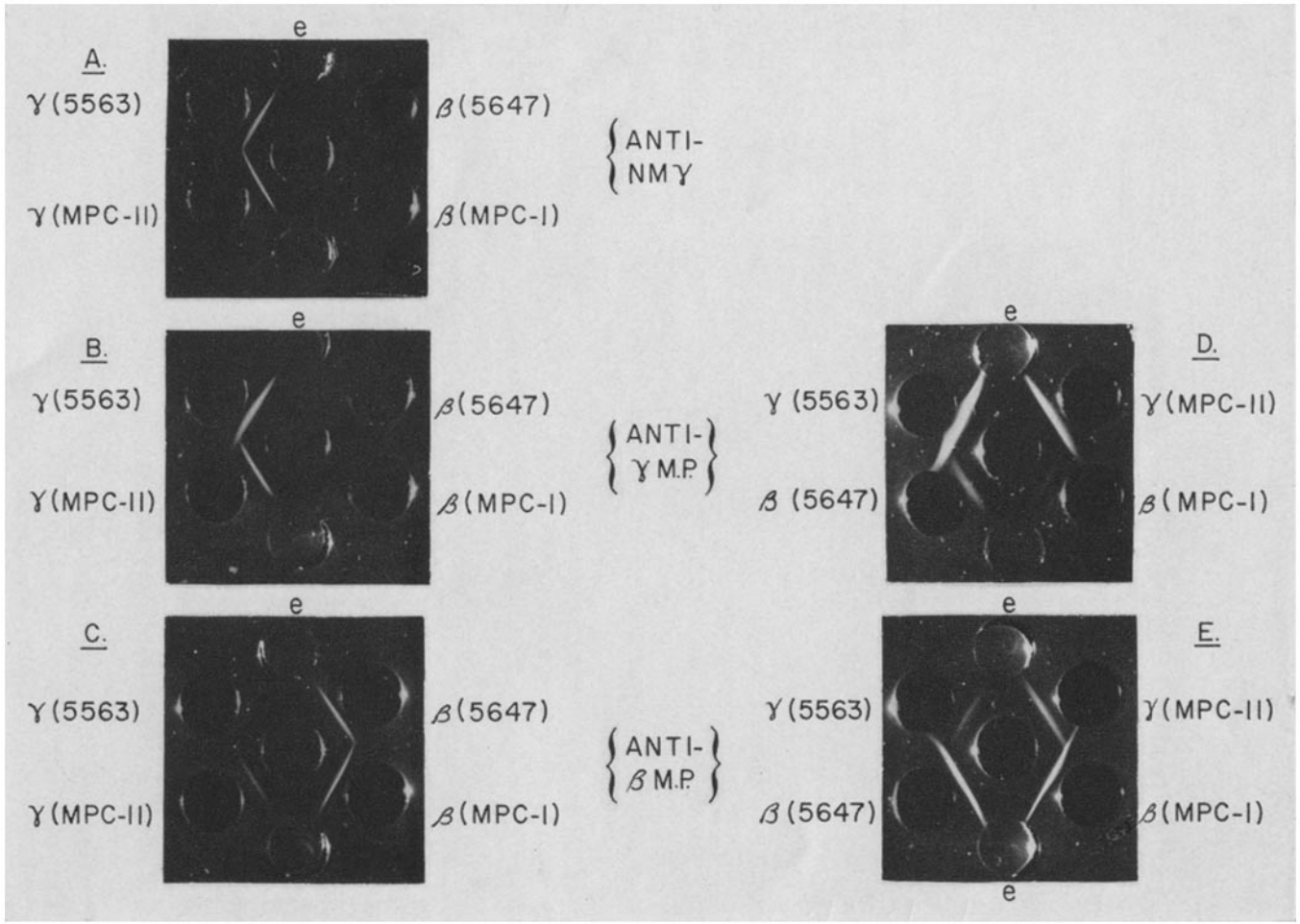

FIG. 7. Ouchterlony double diffusion agar plates employing purified myeloma proteins (0.3 mg/ml) and rabbit antisera to normal mouse gamma globulin (AR 15) in *A*, to 5563 myeloma protein (FR 23) in *B* and (FR 15) in *D*, and to MPC-2 myeloma protein (AR-5) in *C* and *E*. Photographs were taken 24 or 48 hours after filling cups with reagents. Cups labeled *e* were not used in the test.

The antigenic complexity of the globulins was indicated by the studies employing Ouchterlony double diffusion techniques. Six antigenic determinants would account for the distinctive as well as the common antigenic sites observed in Fig. 7 and more antigenic determinants may well be involved. Antigenic differences between individual globulins have already been demonstrated and significant subdivisions within these groups may become apparent with further study. However, the validity of the general division of myeloma proteins into the two groups was supported by observations employing antisera to four different purified globulins, two of the γ-type and two of the β-type.

DISCUSSION

Four classes of proteins, the 6.6 S γ-globulins, the β_{2A}-globulins, the 18 S macroglobulins, and the small, Bence Jones proteins, have been associated closely with plasma cells and related cells, principally on the basis of studies in man. These four classes (or their equivalents) also occur in the mouse. Both 6.6 S and macroglobulin antibodies have been identified in normal mouse serum (18) and Bence Jones proteins were found with a transplantable plasma cell tumor (6). Beta globulins in the mouse, equivalent in many respects to the β_{2A}-globulins of man, are described in the present report.

Mouse myeloma proteins were immunochemically classified as either γ- or β-type on the basis of distinctive antigenic features, and this immunochemical classification has been supported and extended by physicochemical observations which indicate differences between the ultracentrifugal and starch gel electrophoretic behavior and the hexose content of these two groups of globulins (19). The gamma type myeloma proteins were closely related antigenically to the normal 6.6 S globulins which comprise the bulk of the electrophoretic gamma globulin region.

Beta type mouse myeloma proteins are related to a normal serum component, not gamma globulin, which appears to be present in low concentration in normal serum. This component which migrates in the mid-beta globulin region in fresh normal serum of two mouse strains, could not be termed a beta-2 globulin on the basis of electrophoretic mobility. Similarly, most of the myeloma proteins of this antigenic group are of mid- or fast-beta electrophoretic mobility (Fig. 4) and some are alpha globulins. Therefore, the term β-type myeloma protein was used to indicate the predominant electrophoretic properties of this antigenically distinctive group of globulins.[3] It is not intended, however, to imply that all myeloma proteins in each category (γ or β) will conform in all

[3] Potter and Kuff (20) employing immunoelectrophoresis with antisera against tumor microsomal preparations, studied the myeloma proteins associated with 13 tumors in BALB/c mice, and divided the myeloma proteins between two immunoelectrophoretic groups, designated systems I and II. Their systems appear to be based on the detection of the antigenic properties, respectively, of the gamma and beta type myeloma globulins.

features. Clausen *et al.* (11), for instance, have noted a myeloma protein with beta globulin electrophoretic mobility which was antigenically related to the gamma globulins, and could be classified immunoelectrophoretically as a gamma type globulin. A variety of properties are to be expected for the myeloma proteins within each group as, indeed, was the case in the present study.

Beta myeloma proteins are synthesized by the malignant plasma cells. That they do not result from conversion of γ- to β-type elsewhere in the body was shown by *in vitro* isotope incorporation studies with MPC-2 plasma cell tumor slices (21). Since beta type myeloma proteins were shown to be synthesized in plasma cells it is reasonable to expect that the equivalent normal component is similarly synthesized in normal plasma cells.

The evidence that each plasma cell tumor formed only γ- or β-type myeloma protein (never both) would indicate that there may be two types of plasma cells, those forming γ- and those forming β-type globulins. Why there should

TABLE I

Frequency of Two Types of Plasma Cell Tumors

Myeloma protein type	Man (38)*	Mouse (20)*
	per cent	*per cent*
Gamma	76	10
Beta (β_{2A})	24	90

* Number of patients and mouse tumors studied are given in parenthesis.

be two classes of serum globulins formed in plasma cells is unknown. Since these two classes have been found in mouse and man, however, it may be expected that they will be recognized in other species as well.

Other features of plasma cell malignancy may relate to whether a plasma cell tumor produces a γ-type or β-type myeloma protein. It has already been shown that normal gamma globulin catabolism is much more markedly accelerated by plasma cell tumors producing gamma myeloma protein than by those producing a beta myeloma protein in mice (22) and in man (23).

Although the similarity of the two types of mouse myeloma proteins to the two types of myeloma proteins in man has been emphasized, the experience to date indicates one notable difference, *i.e.*, in the frequency with which the two types are found. As shown in Table I, gamma myeloma proteins in man predominate over beta type by a ratio of about 3:1, whereas in the mouse the ratio is more than reversed, 1:9. It is not known whether this species difference reflects some difference in the site at which malignancy develops—intra-abdominally in the mouse and, apparently, in the bone marrow in man— or differences in the conditions leading to malignancy, or some other factor. It seems clear, however, that the frequency of each myeloma protein type does not relate to relative amount of each of the equivalent globulins present

in normal serum. Both man and mouse have much larger amounts of γ- than β (β_{2a})–type globulins in normal serum and in both species most antibodies probably are among the 6.6S γ-globulins.

SUMMARY

The serum myeloma proteins associated with 20 mouse plasma cell tumors in C_3H or BALB/c mice that had proved transplantable were characterized by electrophoretic and immunochemical techniques. Although the myeloma proteins ranged in electrophoretic mobility from gamma to alpha globulins, they could be divided into two groups, the gamma type and the beta type myeloma globulins, on the basis of characteristic immunochemical properties. Gamma type myeloma proteins (5563, MPC-11) showed a close immunochemical relationship to normal mouse gamma globulins.

Eighteen beta type mouse myeloma proteins migrated as beta or alpha globulins on zone electrophoresis. These proteins shared common antigenic features which permitted their recognition, separate from gamma myeloma proteins. The beta type myeloma proteins were shown to be related to a beta globulin component present in normal serum. Strain differences were observed for the normal beta globulin component believed to be formed in plasma cells.

The proteins formed in mouse plasma cells were found to be antigenically complex. Shared antigenic determinants as well as distinctive antigenic determinants were detected when representative myeloma proteins were purified and compared by the Ouchterlony double diffusion technique.

The myeloma proteins associated with each of the transplantable plasma cell tumors in mice are regarded as distinctive and characteristic products of plasma cell metabolism. The variety of myeloma globulins was similar for plasma cell tumors arising in C_3H as well as in BALB/c mice, indicating that differences in mouse strains would not account for the differences among the myeloma globulins. These differences, however, may be due to differences among the normal plasma cells from which the malignant cells are derived. If this is so, the variety of myeloma globulins reflect the variety of plasma cells present normally.

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