Multiple Antigenic Determinants on Macroglobulins.* (28392)

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Although macroglobulins of normal human serum have varied functions(1), there has been no evidence of antigenic individuality among them(2). Some reports have suggested, however, that the macroglobulins of different patients with Waldenström's disease may contain partially specific antigenic determinants(3,4,5) and the results of Boerma and Mandema(4) indicated that at least 2 different antibodies might be formed against individual pathological macroglobulins. This report shows that in both healthy and sick people the serum macroglobulins have various antigenic characteristics and form several distinct immunoelectrophoretic patterns. The latter may be interpreted as indicating either that individual molecules of a single class of serum macroglobulins have several antigenic groupings or that there are several sorts of antigenically distinct macroglobulin molecules in serum.

Materials and methods. Analytical ultracentrifugation, kindly provided by The Institute of Medical Physics, was made at 26°C. Immunoelectrophoresis was made on cellulose acetate(6) using barbital buffer pH 8.6, and ionic strength .075. Electrophoresis was made at 150 volts for 1 hour; the immunodiffusion lasted 48 hours. Precipitin lines were developed with Ponceau S.

The *electrophoretic values* (expressed as percentages of the total protein) of serum obtained from a patient with macroglobulinemia were as follows: albumin, 33.4%; alpha-1 globulin, 4.1%; alpha-2 globulin, 10.3%; beta globulin, 5.2%; gamma globulin, 47.0%. The *analytical ultracentrifuge values* for this serum (diluted 1:10 with 0.85% NaCl) were: 4S., 3.7 g per 100 ml serum (g%); 7S., 1.2 g%; 10S., 0.32 g%; 15S., 7.05 g%; 21S., 1.31 g%; 26S., 0.63 g%; 30S., 0.15 g%. Euglobulin from 20 ml of this serum was precipitated and washed with deionized water, dissolved in 12 ml 0.85% saline and emulsified with 12 ml of complete Freund's adjuvant. Initially and again 3½ weeks later, 10 ml of the emulsion were injected subcutaneously into a 7 lb albino rabbit. The rabbit was exsanguinated 35 days after the last injection and the antiserum stored at −20°C.

When reacted by immunoelectrophoresis with normal human sera, the unabsorbed rabbit serum caused several beta-gamma precipitin lines (Fig. 1). The antiserum was absorbed with serum 59064 (*ultracentrifugal analysis*:† 4S., 1.8 g%; 7S., 13.2 g%; no measurable amount of macroglobulin) in the ratio of 4 parts antiserum to 1 part serum 59064. It may be assumed that the remaining antibodies were directed against serum proteins of higher sedimentation constant than 7S: no precipitin lines were produced with Cohn's fraction II. This absorbed antiserum was used in most of the experiments reported below. Antisera used in a few other experiments were: a horse antiserum to human serum, obtained from the Pasteur Institute (Lot No. 13467) and absorbed in the same way with serum 59064; a dog antiserum to human alpha-2 macroglobulin, obtained from Behringwerke Ag.; and a goat antiserum to human beta-lipoprotein, obtained from Mann Research Laboratories Inc. The sera examined included 12 from healthy medical students, 16 from patients with macroglobulinemia, mesoglobulinemia (7) or both, and 24 from patients with various connective tissue diseases.

Findings and comment. The absorbed antiserum produced the following precipitin lines: A strong, conventional beta-2M line, seen in all sera examined, is here termed beta-2Ma (Fig. 2). A fainter line, beta-

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† Serum 59064 was obtained from a patient with multiple myeloma. Ultracentrifugal analysis was made at 26°C on serum diluted 1:10 in 0.85% saline.

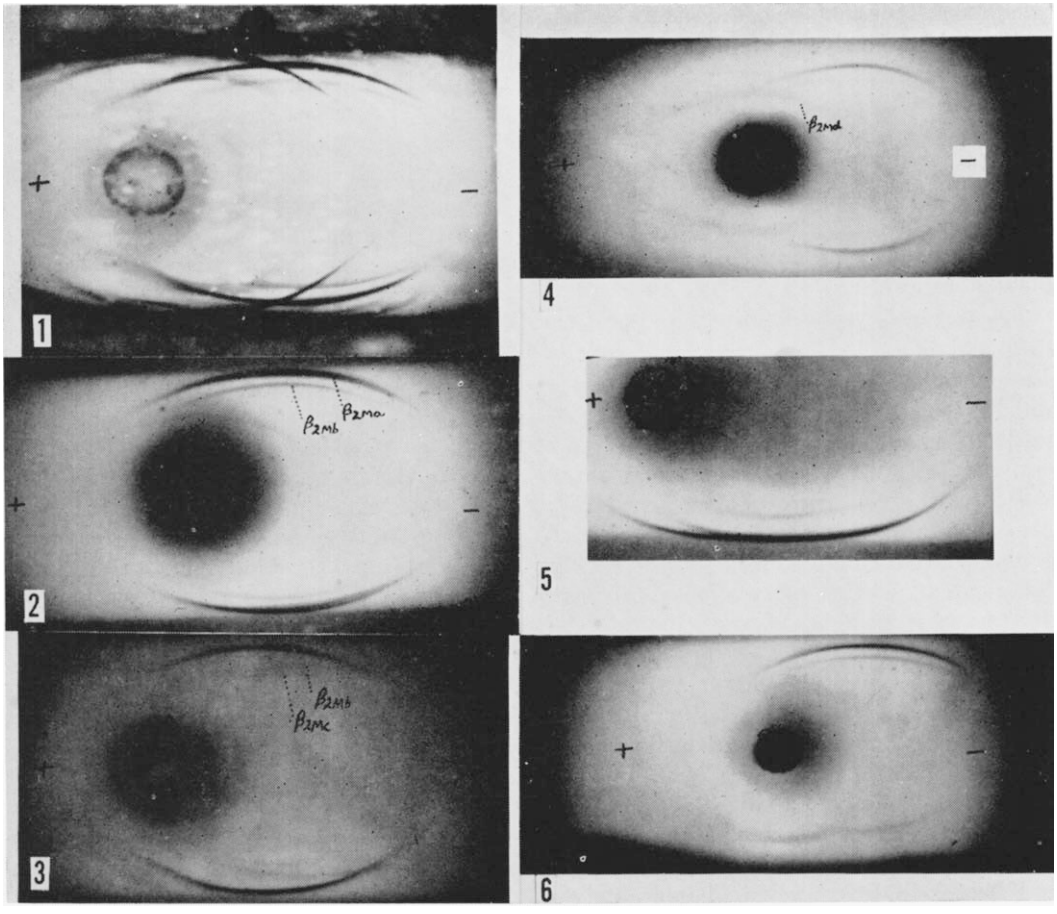

FIG. 1. Unabsorbed rabbit antiserum reacted with normal serum.

FIG. 2. Absorbed rabbit antiserum reacted with normal serum. Beta-2Ma, beta-2Mc, and faint beta-2Md present.

FIG. 3. Absorbed rabbit antiserum reacted with schizophrenic patient's serum. Beta-2Mb fusing with cathodal tip of beta-2Ma. Beta-2Mc present.

FIG. 4. Absorbed rabbit antiserum reacted with normal human serum. Beta-2Md line clearly demonstrated.

FIG. 5. Absorbed rabbit antiserum reacted with rheumatoid arthritis patient's serum. Anodal forking of beta-2Ma line.

FIG. 6. Top: absorbed rabbit antiserum. Bottom: absorbed antiserum from Pasteur Institute producing a precipitin line with a double curvature.

2Mb, having approximately similar arc to beta-2Ma but roughly parallel and internal to it, was seen in all except 2 sera examined (Fig. 2). In many sera the cathodal end of beta-2Mb inclined toward beta-2Ma and occasionally appeared to fuse with it (Fig. 3). Internal to beta-2Mb another faint line, beta-2Mc, appeared in 6 sera (Fig. 3). These 6 sera were from patients with rheumatoid arthritis (2), systemic lupus erythematosus (2), schizophrenia (1), and from one medical student. A fourth, quite strong line, beta-2Md, immediately opposite the serum source and having a reaction of partial identity with beta-2Ma, was present in almost every serum examined (Fig. 4). It was shown by using the appropriate antisera that the beta-2Md line did not give a reaction of immunological identity with alpha-2 macroglobulin or with beta-lipoprotein. Six sera showed a clear forking of the anodal tip of beta-2Ma which was not due to an artifact because it was reproducible on 2 or 3 retests (Fig. 5). These 6 sera were from patients with macro-

globulinemia, dermatomyositis, and rheumatoid arthritis. Lastly, the antiserum obtained from the Pasteur Institute produced, after absorption with serum 59064, a single precipitin line with a double curvature (Fig. 6). The anodal hump of this double curve was not present in the beta-2Ma line; this anodal hump might represent another separate antigenic determinant on the macroglobulin molecule.

The following facts indicate that the beta-2Mb and beta-2Mc lines were produced by macroglobulins: 1) They were closer to the antigen (serum) source than beta-2Ma, suggesting that they were produced by larger serum molecules, by less concentrated serum molecules, or by faster moving antibodies than those responsible for beta-2Ma, which is the conventional macroglobulin line. 2) An apparent reaction of immunological identity was sometimes seen between the cathodal tips of the beta-2Ma and beta-2Mb lines (although this reaction did not occur with beta-2Mc). 3) When the buffer used for both electrophoresis and the diffusion phase contained 0.1 M 2-mercaptoethanol, or when serum was made 0.1 M with respect to 2-mercaptoethanol before immunoelectrophoresis, in some experiments both the beta-2Ma and beta-2Mb lines became fainter and in others they disappeared. That the beta-2Md line was produced by a macroglobulin is suggested by its reaction of partial identity, sometimes with beta-2Ma and sometimes with beta-2Mb. The final proof that the beta-2Ma, beta-2Mb, and beta-2Mc lines were due to serum macroglobulins is that they did not appear after the rabbit antiserum, already absorbed by serum 59064, was further repeatedly absorbed by isolated macroglobulin (kindly provided by Dr. H. H. Fudenberg).

There are three possible explanations for these findings. First, that a single molecular species of macroglobulins present in human sera contains several antigenic sites—at least 4, to account for beta-2Ma, beta-2Mb, beta-2Mc, and beta-2Md; a fifth antigenic determinant could account for the anodal forking of the beta-2Ma line, and yet a sixth might be responsible for the anodal end of the doubly curved beta-2M line seen with the Pasteur Institute antiserum but not with the rabbit antiserum used in these experiments. The second possible explanation is that the macroglobulin peak seen ultracentrifugally contains several immunologically distinct molecules, but that some of these molecules have antigenic sites in common with each other. This might account for the few instances where the cathodal tips of beta-2Ma and beta-2Mb appeared to fuse in a reaction of identity. The existence of several immunologically distinct macroglobulin molecules would be more consistent with the functional heterogeneity of macroglobulins(1), and would also parallel the finding of several 7S gamma globulin precipitin lines(8). The final possibility to be mentioned is that beta-2Mb and beta-2Mc, both closer to the serum source than beta-2Ma, represent macroglobulins of higher sedimentation constant than 19S but present in such small amounts as to be undetectable by usual ultracentrifuge techniques. After normal serum had been enriched in heavy components by means of repeated preparative ultracentrifugation, Wallenius and coworkers(9) could demonstrate trace amounts of 28S and 44S components. This possibility to account for beta-2Mb and beta-2Mc seems rather unlikely because 1) these lines were quite sharply defined, as though present in more than trace amounts, and 2) the beta-2Mc was present in only a few of the sera examined which contained macroglobulins of as many as 5 different sedimentation constants.

Why there has been no previous demonstration of multiple antigenic determinants on macroglobulins of nonmacroglobulinemic sera is not clear. Perhaps the rabbit used for immunization was particularly responsive to these antigens. Or perhaps the macroglobulins in the serum, which had been sent by airmail, had been acted upon by serum enzymes that exposed buried antigenic sites on the macroglobulin molecules. Lastly, if the correct explanation of the lines described above is that they represent multiple antigenically distinct molecules, their demonstration might have resulted from the use of an immunizing serum which contained, in quite

large amounts, 5 classes of proteins having a sedimentation constant above 7S.

Summary. When a rabbit antiserum to human macroglobulin was reacted with human sera by immunoelectrophoresis, several distinct macroglobulin precipitin lines were revealed. Internal to the major beta-2M line all sera showed a second, and some a third, line. A fourth macroglobulin line appeared opposite the serum source in most sera examined. The major macroglobulin line was forked at its anodal end in some sera. The major macroglobulin line had a single curvature when produced by the rabbit antiserum used in most of these experiments, but had a double curvature when produced by a horse antiserum. These findings could be due to multiple antigenic sites on macroglobulins of a single molecular species, or to antigenically distinct macroglobulin molecules.

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Oxidative Responses *in vivo* of 2,4-Dinitrophenol and Carbonyl Cyanide-p-Trifluoromethoxyphenylhydrazone. (28393)

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A recent report has indicated that ring-substituted derivatives of carbonyl cyanide phenylhydrazone are potent uncouplers of oxidative phosphorylation in both animal and plant mitochondrial systems(1). The most effective of the compounds reported was carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (p-CF_3O-CCP), reported to be about 1000 times as active as 2,4-dinitrophenol (DNP) *in vitro*. It was, therefore, of interest to measure the general oxidative response of intact laboratory animals to p-CF_3O-CCP.

The present report contains results obtained from mice and rats in a comparative study of metabolic effects of DNP and p-CF_3O-CCP. Measurement of both $C^{14}O_2$ resulting from oxidation of C^{14}-2-acetate and total expired CO_2 was used to determine general oxidative responses to uncoupling agents and as an indication of metabolic rate (MR) respectively. Stimulatory effects of DNP have been observed using similar methods(2) and oxidative uncoupling effects of DNP are well established(3).

Materials and methods. Ad libitum fed male albino mice and rats were used. Mice weighed 27-32 g (Rockland Farm Swiss mouse ancestry) and rats weighed 165-175 g (Charles River). Fresh solutions of uncoupling agents were made daily and injected intraperitoneally (IP) into mice or rats in 0.2 ml volume; control animals received the appropriate vehicle alone. Solutions of DNP in 0.1 N $NaHCO_3$ were given at a dose of 15 mg/kg while p-CF_3O-CCP* (11 mg/kg) was administered in methyl cellulose solution (0.25% aqueous vehicle). These doses represented approximately 20% of the acute

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