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Physical Heterogeneity of Bovine Gamma Globulins: Gamma-1M Globulin Electrophoretic Heterogeneity.* (29624)

FREDERICK A. MURPHY,† OLE AALUND AND JOHN W. OSEBOLD
(Introduced by Norman F. Baker)
Department of Veterinary Microbiology, School of Veterinary Medicine, University of California, Davis

The distributions of 7S gamma globulins mediating particular primary and secondary antibody functions, such as binding of antigen(1), fixation of complement(2) and fixation to skin(3) may be heterogeneous within the electrical charge continuum of this protein taken as a whole. The length of the immunoelectrophoretic arc of gamma-1 macroglobulin (gamma-1M) suggests the presence of a continuum of molecules differing in net electrical charge in a manner analogous to the spectrum of 7S gamma globulin molecules. In addition to electrical charge heterogeneity, the identification in macroglobulin preparations of very large molecules by ultracentrifugal analysis (26S, 32S)(4) provides evidence of a polymer type heterogeneity perhaps analogous to the 7S gamma-1A globulin polymer heterogeneity. However, no experimental definition of the extent of gamma-1M globulin electrophoretic heterogeneity is available, nor has there been any reported success in attempting to associate particular antibody activities with gamma-1M globulins having particular mobility properties. Data are presented here to contend that in the bovine species at least one biological activity, the ability to fix complement in the presence of an appropriate antigen, is associated with gamma-1M globulin molecules whose mean net electrical charge differs from that of the protein as a whole.

Materials and methods. Sera. Serum samples were taken from adult cattle, 1243 and 1244, experimentally infected with *Anaplasma marginale*. Samples were pooled from those days early in the disease where DEAE Sephadex chromatography of whole serum and subsequent serological assay of fractions indicated strong, complement-fixing antibody response exclusively in the macroglobulin. This was manifest by high antibody titer in DEAE peak III fractions together with failure to obtain any fixation with DEAE peak II protein—the fast 7S gamma globulin containing antibody activity in later response(5).

Gel filtration. Bovine sera were fractionated on Sephadex G200 (Pharmacia Fine Chemicals, Inc., New York) in a column 7 × 70 cm containing approximately 150 g (dry weight) of the cross-linked dextran, according to the technique of Flodin for large columns(6). Elution buffer consisted of 0.1M Tris + 1M NaCl at pH 8.0. Protein appeared in the eluent in 3 main peaks as has been shown with human serum. Analysis of the distribution of proteins in these

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† Present address: Virology Section, Communicable Disease Center, Atlanta, Georgia.

peaks was accomplished using immunoelectrophoresis.

Preparative zone electrophoresis was performed according to the methods of Müller-Eberhard(7) and Fahey and McLaughlin(8). Pevikon C-870 (copolymer of polyvinyl chloride and polyvinyl acetate) (Stockholm Superfosfat Fabrika AB, Stockholm) particles were suspended in a buffer of 0.075M barbital, pH 8.6, to form blocks with dimensions of 39 × 6.5 × 1.3 cm. Electrophoresis was allowed to proceed at 200 V (constant) for 40 hr at 4°C, using a brom-thymol-blue labeled albumin reference to estimate the extent of migration.

Agar electrophoresis was performed on glass plates (3.25 × 4 in lantern slides) in a 1 mm deep layer of 1.5% Ionagar #2 (Consolidated Laboratories, Chicago Heights) made up in 0.075M barbital buffer at pH 8.6.

Immunoelectrophoresis was performed according to the micro-modification of Scheidegger(9) using the same lantern slide format to contain four patterns in parallel. Rabbit anti-bovine macroglobulin (anti-Sephadex G200 peak I protein) was used.

Complement-fixation serology for determination of anti-*Anaplasma* antibody activity was performed according to the current protocol of the Agricultural Research Service, U. S. Department of Agriculture(10).

Protein determination. Optical density at 280 mμ was used to estimate protein concentration in gel filtration fractions. The Lowry method(11) (modified only in that twice the standard volume of the $CuSO_4$-Na_2CO_3 solution was used per test) was used to determine protein content of electrophoretic fractions. A crystallized bovine plasma albumin standard was used; values given for protein concentration of zone electrophoresis fractions are in mg/ml of bovine albumin.

Results. Forty to 42 ml serum pools, taken from animals 1243 and 1244 during the early phase of antibody synthesis, were applied to columns of Sephadex G200 for gel filtration. Effluent fractions were collected; localization of gamma-1M globulin and alpha-2M globulin in peak I was confirmed by immunoelectrophoresis. The fractions making up this peak in each case were pooled (shaded area of Fig. 1) and concentrated by dialysis against 20% polyvinylpyrrolidinone (PVP) to a total volume of 5 ml. This volume was applied to a trough cut into a Pevikon block 7 cm from the cathodal end for electrophoretic fractionation. Following electrophoresis, the blocks were sliced transversely into 1 cm segments; each segment was eluted with 5 ml of 0.85% NaCl. The liquid phase was separated by filtration through a sintered glass funnel, followed by centrifugation to remove remaining particles of the Pevikon. Each fraction, now representing one cm of the Pevikon bed was analyzed for protein content, dialyzed against 0.85% NaCl and titrated for complement-fixing antibody activity (Fig. 1). The mean distribution of complement-fixing antibody activity was skewed to the cathodal side of the distribution curve of the gamma-1M protein, the electrophoretically slow peak. This eccentricity of the mean distribution of antibody *vs* protein was 1.1 cm for animal 1243 and 2.0 cm for animal 1244 as measured by constructing the mean of each antibody and protein peak from its center of symmetry. In Fig. 1, "A" denotes the center of symmetry of the complement-fixing activity peaks; "B" the center of symmetry of the protein peaks. Thus, the constructed means confirm visual observation that antibody activity is associated with molecules of macroglobulin whose mean electrophoretic mobility is slower than that of the protein as a whole. The possibility of the presence of alpha-2M globulin in the anodal end of the gamma-1M globulin peak as a diluting factor accounting for the skew of antibody activity toward the cathodal end, was eliminated by immunoelectrophoretic confirmation of the efficiency of the electrophoretic separation of the two proteins. The above experiments on each serum pool were duplicated.

Nine fractions from the electrophoretic separation of the G200 peak I protein from each animal were electrophoresed in agar on lantern slides. Following electrophoresis these slides were immediately immersed in acidified

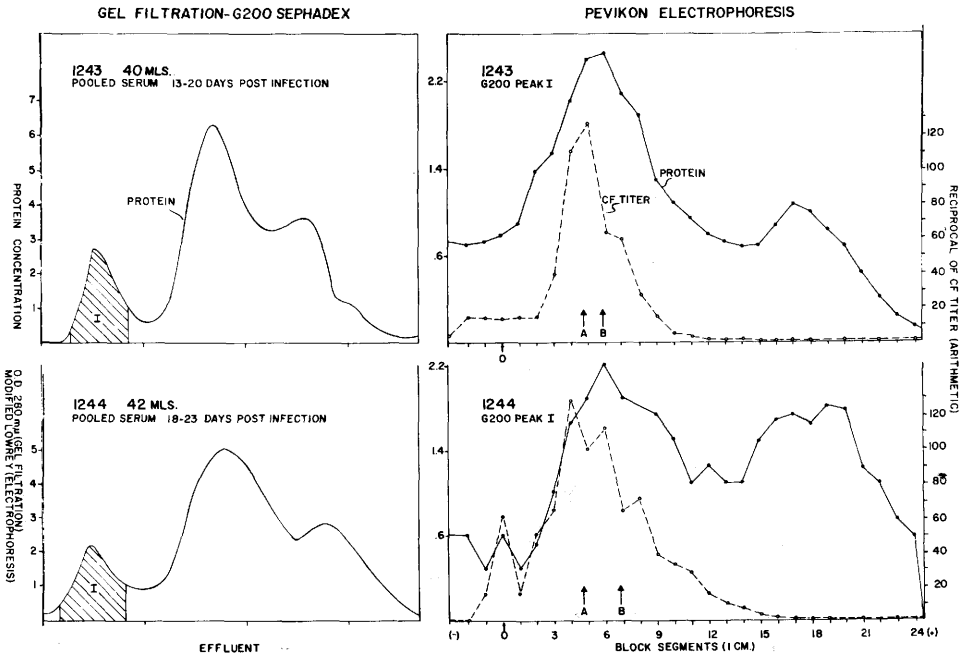

FIG. 1. Gel filtration chromatograms of bovine sera 1243 and 1244 (left). Zone electrophoresis in Pevikon of protein eluted as peak I from gel filtration of same sera (right). A, center of symmetry of complement-fixing (CF) antibody titer peak; B, center of symmetry of the associated protein peak (gamma-1M).

Amido Black stain (0.1%) for 30 min, cleared for several hours in 2% acetic acid and photographed against a bright field (Fig. 2—top). It can be seen that a good separation of the 2 macroglobulins, alpha and gamma, had been accomplished by the zone electrophoresis and that the alpha-2M globulin appears to be electrophoretically homogeneous since fractions even 6 cm apart have virtually identical mobilities (segments 15-24). This is consonant with the behavior of alpha-2M globulin in starch gel electrophoresis. On the other hand, a whole spectrum of mobilities is seen in the fractions from the gamma-1M globulin area (segment 0-12). The gamma-1M globulin peak on Pevikon zone electrophoresis represents a real continuum of molecules of different mobilities rather than a random normalized distribution of molecules of the same mobility as shown to be the case with the alpha-2M globulin. It would appear in this case that the skew of the antibody titer peak toward the cathode is indeed representative of association of such activity with molecules having at least this one electrophoretic characteristic distinct from the mean of the whole population of the protein. Immunoelectrophoretic analysis of these same Pevikon fractions (Fig. 2—bottom) likewise showed the separation of the 2 macroglobulins, the virtual freedom of these fractions from contamination with other serum components, the homogeneity of the alpha-2M globulin and the electrophoretic heterogeneity of the gamma-1M globulin.

When analytical ultracentrifugation of those Pevikon fractions from the gamma-1M area (from animal 1244) was performed, the sedimentation coefficient of the main component was $18.60S_{20,w}$ and that of a minor component $25.02S_{20,w}$(12). The 18S component represents the gamma-1M globulin and the heavier component most likely the first polymer of this protein.

Discussion. When bovine serum macroglobulins, separated from the rest of the serum components by gel filtration on Sephadex G200, were electrophoretically spread through 28 cm of a Pevikon block, such that the gamma-1M globulin was itself spread through

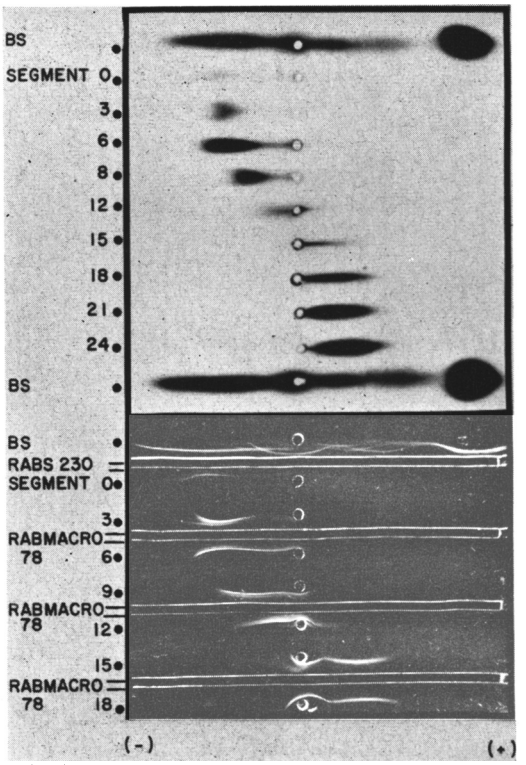

FIG. 2. Agar gel electrophoresis and immunoelectrophoresis of eluent from segments from the electrophoretic separation of macroglobulins of animal 1244 in Pevikon. BS, bovine serum. RABS, rabbit anti-bovine serum. RABMACRO, rabbit anti-bovine macroglobulin.

16 segments (each 1 cm wide), the demonstrated heterogeneous distribution of complement-fixing antibody activity was small indeed. Even with this extensive separation, the center of symmetry of the complement-fixing antibody titer peak was skewed only 1.1 cm in one case and 2.0 cm in another toward the cathode from the center of symmetry of the gamma-1M protein peak. The smallness of these differences notwithstanding, it is contended that this skew is real and that it is indicative of the association of this particular antibody activity with macroglobulin molecules whose mean electrophoretic mobility is slower than that of the total population.

These findings must be discussed in light of two hypotheses. "Influence of the specificity of the immunizing antigen on the electrophoretic mobility of . . . antibodies produced"(14) is the basis of a concept well documented in the case of 7S gamma globulin antibodies(13,14) but untested with macroglobulin antibodies. Considering the antigenic mosaic of the rather complex organism, *A. marginale* presented to the host animal, it is impossible to elaborate upon the skew of the complement-fixing antibody titer peak noted here as a specific effect of a particular antigenic stimulus. Influence of the specificity of the immunizing antigens upon the mobility of antibodies synthesized is certainly not denied by these experiments, but another hypothesis may be offered for discussion. Since during the early course of antibody response, those molecules specifically synthesized within a particular physicochemically distinct gamma globulin group (gamma-1M in this case) are of younger than mean age, the above data may be explained by postulating that new molecules are less electronegative than older ones where effects of wear and tear might alter net charge. The effect of sialic acid bound to glycoproteins (*e.g.* gamma-1M globulin) upon electrophoretic mobility(15) and the ease with which this residue may be split off physiologically exemplifies the feasibility of such alteration. This might account for skews of biological activity peaks of small magnitude but by no means is such a hypothesis offered in explanation of the gross electrophoretic heterogeneity of 7S gamma globulins which has been shown to be a reflection of major electrical charge differences between molecules of the various subgroups within the continuum.

Summary. In the bovine species, gamma-1M globulin occurs as an electrophoretic continuum with most molecules of near mean mobility. No electrophoretic sub-groups of particularly high concentration, as described for 7S gamma globulins, were noted. Complement-fixing antibody activity was associated with bovine gamma-1M globulin molecules whose mean electrophoretic mobility was slower than the mean mobility of the protein as a whole. The marginal extent of this manifestation argues against anticipation of as heterogeneous a distribution of particular biological activities within the gamma-1M glob-

ulin spectrum as has been shown to be the case with 7S gamma globulins.

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Interferon Production *in vitro* by Cells Infected with the Murine Salivary Gland Virus.* (29625)

DONALD HENSON AND ROGER D. SMITH (Introduced by G. M. Hass)
Division of Pathology, Presbyterian-St. Luke's Hospital, Chicago, Ill.

Infection with the murine salivary gland virus (MSGV) characteristically results in a chronic inapparent infection evidenced by persistent demonstrable virus and intranuclear inclusions in the salivary glands of mice(1,2). On this basis it seems, therefore, that MSGV may serve as an ideal experimental tool for investigation of viral latency. Initial attempts to define the host-virus relationship have led to demonstration of an interfering substance produced *in vitro*.

In this report, the effect of this interfering substance on a heterologous virus as well as some of its properties are described. The results indicate that this interfering substance has many properties similar to those of interferon(3,4).

Materials and methods. Cell cultures. "L" cells, Strain 929, were serially propagated in 1-liter bottles using a growth medium composed of 95% Earle's balanced salt solution, amino acids and vitamins specified by Eagle (5), 5% calf serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml Kantrex, and 10 μg/ml Fungizone. Monolayers were prepared by versenating the cells, resuspending in medium, and adding 1×10^6 cells to each 60 mm plastic Petri dish. Monolayers were used after 24 hours of incubation in an atmosphere of 5% CO_2 in air at 37°C.

Mouse embryonic fibroblasts, prepared by the method of Youngner(6) using 13-15 day-old embryos from SWR/J mice, were grown in 1-liter bottles using the same medium as for the "L" cells. Monolayer cultures in 60 mm Petri dishes were prepared by trypsinizing densely populated bottle cultures, resuspending the cells in medium, and then adding 1.5×10^6 cells to each dish. Cultures were used 24 to 48 hours later.

Viruses. Mice chronically infected with MSGV were obtained through the courtesy of Dr. Henry Pinkerton. Using a procedure similar to that reported by Smith(7), bottle cultures of mouse embryonic fibroblasts were

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