Evidence for Anti-Gm(a) Antibody in γA Immunoglobulins.* (31684)

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Human antibodies against human γ globulin occur in a number of forms, including rheumatoid factors in patients with rheumatoid arthritis and other diseases(1). In addition, some sera from non-rheumatoid patients have anti-γ globulin antibody with specificity for one of the genetic Gm factors occurring on the H polypeptide chain of γG globulin. Such anti-Gm activity is most frequently found in molecules of the γM globulins(2,3) but γG globulins with this activity have been reported(2,4). The finding of anti-Gm activity in multiple immunoglobulin classes is in keeping with the concept that such activity is antibody, a concept supported by demonstration of their production following exposure to genetically foreign γ globulin through transfusion or placental transfer(2,5). This report presents data from studies of a serum (serum F.S.) which strongly suggest localization of anti-Gm(a) activity to the γA immunoglobulin class.

Materials and methods. Gm specific hemagglutination. Anti-Gm agglutinating activity against human O, Rh_0 cells coated with incomplete anti-Rh (anti-D) antibodies was assessed by techniques previously reported (2). A number of incomplete anti-Rh antibodies useful in typing for various Gm factors, particularly Gm(a) (anti-D's 2368, 16, 35, 47, 49, Ri) were used. Gm specificity was assigned by using whole sera of known Gm phenotype in standard inhibition of agglutination techniques.

Immunoprecipitin studies. Double diffusion in agar was performed as outlined by Ouchterlony(6). Tubes in precipitin curves were made to constant volume and held at 5°C for 3 days. Precipitates were washed 3 times in ice cold physiologic saline, dissolved in a constant volume 0.1 N NaOH, and protein concentrations determined by the Folin Ciocalteau technique(7). Absorption with precipitating antisera at fixed ratios was accomplished in comparable manner. All antisera against human immunoglobulins were raised in rabbits by immunization with human Cohn fraction II (anti-γG), isolated myeloma proteins (anti-γA) and Waldenstrom macroglobulins (anti-γM). After initial immunization, anti-γA antisera were broadened by stimulation of rabbits with pools of 10 γA myeloma proteins and normal γA globulin isolated from whole serum after the method of Tomasi(8). Similarly, anti-γM antisera were broadened by final stimulation with a pool of 6 Waldenstrom macroglobulins. Specificity of antisera was selected, when necessary, by appropriate absorption with Gm(a—) human serum proteins as follows: anti-γG with pepsin digested human Cohn fraction II, anti-γA and anti-γM with human cord serum. Specificity was verified by double diffusion in agar and immunoelectrophoresis against whole human serum, human fraction II, isolated myeloma proteins and Waldenstrom's macroglobulins. Rabbit antisera were absorbed with human Rh_0 cells prior to reaction with anti-Gm(a) sera to be used in hemagglutination tests.

Separation of immunoglobulins. Chromatography on DEAE cellulose was accomplished after the method of Tomasi(9), starting buffer 0.03 M pH 7.5 phosphate and final buffer 0.3 M pH 4.5 phosphate in a continuous gradient. Serum fractionation on Sephadex G-200 utilized pH 8.0 0.2 M tris. Following chromatography, physiologic pH and ionic strength were established in each fraction prior to testing in hemagglutination systems(10). Sucrose density gradients were performed as described by Kunkel(11), utilizing a continuous gradient made from 10% and 40% sucrose solutions in isotonic sodium chloride. Bacterial alkaline phosphatase (Nutritional Biochemicals Corp.) was utilized as a marker in some experiments with the su-

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TABLE I. Agglutination of Rh(+) Erythrocytes Coated with Anti-D Ri by Serum F.S.: Inhibition by Isolated γG Globulins.

Phenotype of inhibiting γG globulin	Concentration inhibitor γ globulin (mg/ml)							
	1	.5	.25	.12	.06	.03	.01	Sal.
Unaggregated Gm(a+b—f—x—)	0	0	0	0	0	1	2	2
" Gm(a—b+f+x—)	2	2	2	2	2	2	2	2
Aggregated Gm(a—b+f+x—)*	2	2	2	2	2	2	2	2

* This preparation strongly inhibited hemagglutination by a number of rheumatoid arthritis sera.

crose gradients, and its activity assessed by dephosphorylation of para-nitrophenylphosphate spectrophotometrically assessed at 280 mμ after the method of Garen and Levinthal (12). Gamma globulins from individual donors were concentrated by zone electrophoresis in starch(13). Reduction with 0.1 M β mercaptoethanol was performed as previously described(2). Aggregation of γG globulin was achieved by heating at 61-63°C for 11 minutes.

Results. Studies on Gm specific hemagglutinating antibody. The ability of serum F.S. to agglutinate human Rh+ erythrocytes coated with a variety of incomplete anti-Rh antibodies was studied. Two observations were documented. First, in no instance was a prozone seen in these agglutination reactions, though all reactions were done in serial 2-fold dilutions beginning with undiluted serum. Secondly, all hemagglutinating activity in serum F.S. was specific for the Gm(a) genetic factor as shown in the following ways. Only those anti-D coats known to react with anti-Gm(a) agglutinators were agglutinated by this serum. All agglutination was inhibited by 30 Gm(a+x+) and Gm(a+x—) sera and not even partially inhibited by 25 Gm(a—) sera in tests with each of 6 different Gm(a+) anti-Rh antibodies. It should be noted that these studies included two anti-D coats which are Gm(a+) and which detect most rheumatoid factors (Ri, 47). In both instances total inhibition of hemagglutinating activity was attained with Gm(a+) sera, and none with Gm(a—) sera. As shown in Table I, γG globulin isolated from a Gm(a+) donor strongly inhibited agglutination of erythrocytes coated with Gm(a+) incomplete anti-Rh antibodies, while Gm(a—) γ globulin—aggregated or unaggregated—failed to inhibit such agglutination. Identical specificity data were obtained on serum A.S., an anti-Gm(a) agglutinator of comparable titer originally supplied by Dr. M. Harboe (serum Smejsa in ref. 24). Serum A.S. was used in all subsequent studies as one form of control for the behavior of serum F.S.

Susceptibility of anti-Gm(a) activity to reduction by β mercaptoethanol. Treatment of sera F.S. and A.S. with 0.1 M β mercaptoethanol reduced the titer of anti-Gm(a) activity in both instances. Control titers of 1:256 were present in both sera. Following reduction, activity in serum F.S. was reduced to 1:4 (corrected for dilution) while no activity was detectable after reduction of serum A.S. Variation in reduction time of 12 to 48 hours caused no change in these findings.

Localization of Gm(a) specific agglutinating activity. In order to localize anti-Gm(a) agglutinating activity within the immunoglobulin classes, the following studies were performed. In both sera chromatographic fractionation on DEAE cellulose revealed anti-Gm(a) agglutinating activity only in those fractions eluted after the albumin peak, an area shown to contain both γM and γA immunoglobulins. Similarly, chromatographic separation of this serum on Sephadex G-200 at pH 8.4 eluted agglutinating activity in the first major peak with the 19S γM globulins. Previous studies with columns of this size had indicated good resolution of 7S from 19S globulins, but no separation of 19S from intermediate sized molecules in a single passage.

Experiments using sucrose density gradient ultracentrifugation gave striking results, presented in Fig. 1. Experiments with serum F.S. were each controlled with serum A.S., both sera being run simultaneously in different buckets of the same rotor. When ultra-

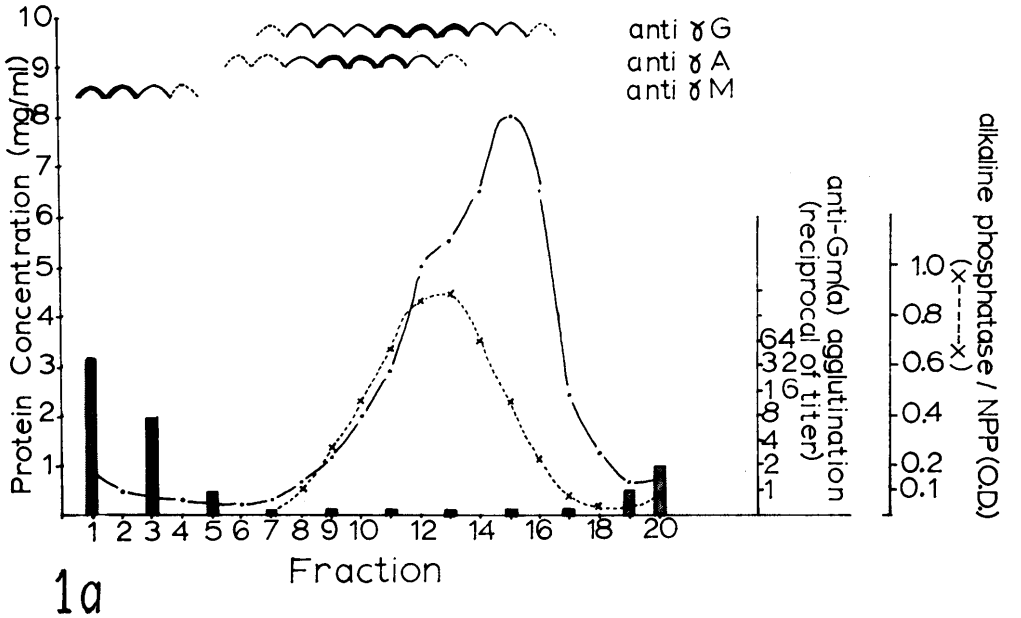
centrifugation was carried on for 18 hours, activity in control serum A.S. (Fig. 1a) was limited to the bottom tube fractions where only γM globulins were detectable by diffusion in agar against specific antisera. Peak activity was at the bottom and significant activity occurred in the final, or blow-out fractions (fractions 19 and 20). Under these conditions peak anti-Gm(a) activity in study serum F.S. (Fig. 1b) is in the region intermediate between the 19S and 7S peaks, clearly lower than peak activity of the alkaline phosphatase marker which has been reported to have an uncorrected S rate of 6.3 (12). Peak anti-Gm(a) activity was above the area of identifiable γM globulins, and while some activity was present in lower portions on the gradient, none appeared in the blow-out fractions. The abnormal configuration of the sucrose density gradient protein curve with serum F.S. is due to the large amount of 7S sedimenting material which it contains, as verified by analytical ultracentrifugation.

To characterize further the type of globulin carrying anti-Gm(a) activity in these sera, absorption experiments were performed. In each instance, quantitative precipitin curves were performed with serum F.S. and each of the antisera to γG, γA or γM globulin. A ratio of antiserum to test serum was chosen in each instance which was in slight antibody excess. Absorptions were carried out at these ratios on both sera F.S. and A.S., and anti-Gm(a) agglutinating activity was titered on the resulting supernatant. In addition, absorption of serum A.S. (the control serum) was performed at ratios determined by its own precipitin curves with the various antisera, resulting in qualitatively identical results. As controls, agglutinating sera were also absorbed with normal rabbit serum at the same ratios. A typical precipitin curve is presented in Fig. 2, showing the reaction between serum F.S. and anti-γA globulin serum G-45; the arrow marks the ratio used for subsequent absorption experiments. The results of anti-Gm(a) hemagglutination tests on the absorbed sera are summarized in Table II. As can be seen, titers when each serum was absorbed with normal rabbit serum or the same volume ratio of antiserum agreed within 2 two-fold dilutions except in two instances. All detectable anti-Gm(a) activity was absorbed from serum A.S. by anti-γM antiserum, representing a 7-tube difference from the control (absorbed with normal rabbit serum) titer. Similarly, detectable anti-Gm(a) activity from serum F.S. was absorbed by anti-γA antiserum, representing a 6-tube difference from the control titer. Variations in the control titers presented in Table II are due to the varying volume ratios at which absorptions were performed. Identical experiments were performed with another anti-γM and anti-γA antiserum, and qualitatively identical results were obtained. Both of these antisera were weaker, however, and demanded larger antiserum to agglutinating serum ratios to achieve absorption at equivalence. Though total absorption of activity was achieved in both instances, the difference between control and absorbed anti-Gm(a) titer was less dramatic than the data presented in Table II.

Discussion. The foregoing data would seem to establish that anti-Gm(a) activity in serum F.S. resides in a different type of immunoglobulin molecule than that supporting anti-Gm(a) activity in serum A.S. This is supported by different position of sedimentation in the sucrose density gradient and differences in absorption by rabbit antisera specific for the different immunoglobulin classes. That at least a significant percentage of the anti-Gm(a) activity in serum F.S. is carried by γA globulin molecules is strongly suggested by these data. Gamma A globulins of higher sedimentation coefficient than 7 are reduced to a 7S unit by reduction with 0.1 M β mercaptoethanol(14), though loss of activity following such treatment does not differentiate activity in γA from that in γM immunoglobulins which are also susceptible to reduction at this concentration of β mercaptoethanol(15). Sephadex chromatography indicated that molecules supporting anti-Gm

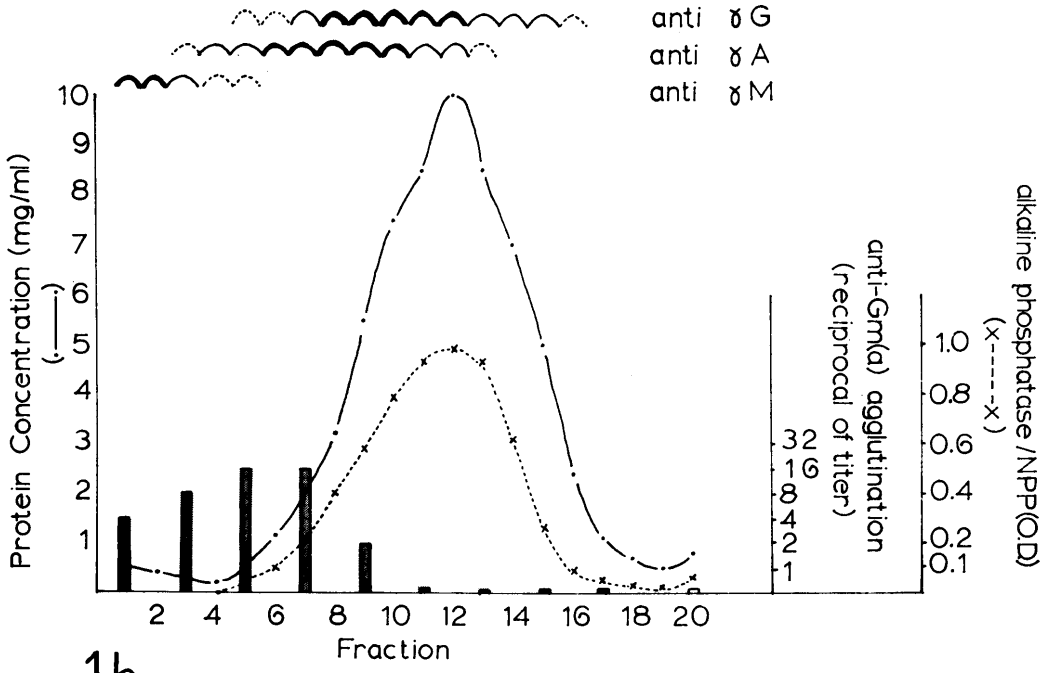
FIG. 1a. Sucrose density gradient on serum A.S. Specific anti-Gm(a) agglutination titers are shown by cross hatched bars; location of immunoglobulin classes are indicated at top of figure by arcs of reaction with specific rabbit antisera. 1b. Sucrose density gradient on serum F.S.

Sucrose Density Gradient: Serum A. S.



1a

Sucrose Density Gradient: Serum F. S.



1b

TABLE II. Anti-Gm(a) Agglutinating Activity Following Absorption by Rabbit Antisera Specific for Different Human Immunoglobulins.

Anti-Gm(a) serum	Titer unabsorbed	Absorbing rabbit serum* Anti-γG (Rab. 22)	Anti-γM (Rab. G-48)	Anti-γA (Rab. G-45)
F.S.	1:256	1:8 (1:16)†	1:64 (1:128)	0 (1:32)
A.S.	1:256	1:4 (1:16)	0 (1: 64)	1:32 (1:16)

* Rabbit antisera absorbed with Rh_0 red cells; all titers recorded as serial 2-fold dilutions from the absorbed anti-Gm(a) serum.

† Titers in parentheses represent those following absorption with same volume of normal rabbit serum.

(a) activity in serum F.S. were larger than 7S γG globulin, and this was confirmed by findings in sucrose gradient ultracentrifugation. The latter technique clearly demonstrated that under these conditions, the major part of anti-Gm(a) activity in F.S. assumed a position intermediate between the 7S and 19S regions, and coincident with the area in which maximal lines were elicited in agar double diffusion tests with specific anti-γA antisera. Antibody activity in this region of sucrose gradient has been shown by Kunkel and Rockey to reside in polymers of γG globulin or intermediate sized γA globulins (16). Absorption of sera F.S. and A.S. with antisera specific for the different human immunoglobulins indicates that anti-Gm(a) activity in both is not antigenically γG globulin. This is in agreement with absence of anti-Gm(a) activity in the γG globulins eluted from DEAE cellulose, Sephadex G-200 and the absence of significant amounts of activity in the 7S region of the sucrose density gradient. Anti-Gm(a) activity in serum A.S. was limited to the 19S region of the sucrose gradient, and was absorbed only by antiserum specific for the γM globulins. Anti-Gm(a) activity in serum F.S., however, was selectively absorbed only by antiserum which detects γA but not γG or γM globulins. The failure of anti-γG and anti-γM antisera to absorb anti-Gm(a) activity from serum F.S., though comparable amounts of protein were precipitated, mitigates against absorption with anti-γA antiserum being a non-specific, or co-precipitation phenomenon.

Appropriate reagents were not available to test the possibility that anti-Gm(a) activity in serum F.S. resides in neither γG, γM nor γA globulins, but rather in the recently described γD globulin(17), or in immunoglobulins associated with reaginic activity as described by Ishizaka *et al*(18). Certain evidence makes these possibilities unlikely, however. After the methods of Martin and Ames(19), assuming a common partial specific volume for the various proteins involved and assuming linear movement of proteins with time for gradients used in these studies, one may calculate from Fig. 1b that peak anti-Gm(a) activity in serum F.S. has an S rate of 11.0 based upon position of the alkaline phosphatase marker at S = 6.3, or 11.8 based upon position of the γG globulin peak at S = 6.8. Peaks of γG globulin and alkaline phosphatase could not be distinguished in the sucrose gradients used here. Both of these values are significantly greater than the gradient determined S rate of 8.0 to 8.3 offered for reaginic immunoglobulin by Ishizaka *et al* (18), and compatible with the figure of 11.1 for the sedimentation coefficient of γA isoagglutinin found by these workers in the

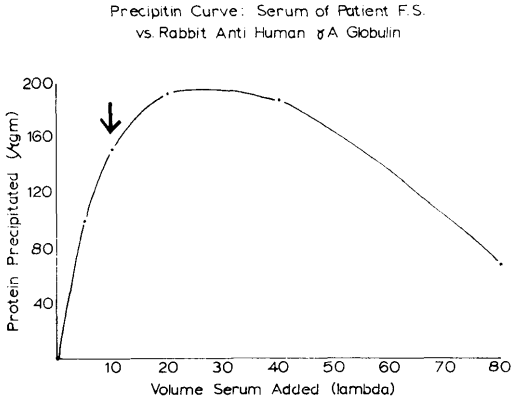


FIG. 2. Precipitin curve: serum F.S. against absorbed rabbit anti-human γA antiserum G-45. Arrow indicates point at which activity absorption experiments were performed.

sucrose density gradient. These figures are even more strikingly different from the S rate of just over 7 found by Rowe and Fahey for the γD immunoglobulins(17).

Though it would seem most anti-Gm(a) activity in serum F.S. resides in γA molecules, some may reside in the other immunoglobulins. Failure to remove all activity by reduction with 0.1 M mercaptoethanol could be due to activity in γG or in 7S γA globulins. Similarly, data from studies with the sucrose density gradient are compatible with some activity in γM globulins or in γA globulins larger than 11S in sedimentation rate. Absorption studies offer no evidence on this point, as absorption of a small percentage of total activity would be missed. Similarly, small amounts of residual activity after absorption could be obscured by the dilution necessary in absorption experiments.

Anti-Gm agglutinators found in the serum of patients without rheumatoid arthritis have characteristics which distinguish them from those found in serum of patients with rheumatoid arthritis(20,21). These include lack of a prozone, specificity only for a single Gm character, and their failure to be inhibited by heat aggregation of γ globulin lacking the Gm character for which they are specific, all characteristics of anti-Gm(a) activity in serum F.S. studied here. It has recently been shown that this type of Gm agglutinator may arise in man as a result of immunization by genetically foreign γ globulin administered by transfusion or by placental transfer from the mother(2,5). Serum F.S. in this study was obtained from a patient with keratoconjunctivitis sicca syndrome but without past or present arthritic complaints. Though a significant percentage of such patients may have rheumatoid factors in their serum(22), it is clear that the characteristics of the anti-Gm activity in serum F.S. are of the type seen in non-rheumatoid individuals, as verified by the characteristics listed above. This is supported by the absence of anti-γ globulin agglutinating activity other than anti-Gm(a) which could be detected in the anti-Rh/Rh agglutinating technique using anti-Rh antibody Ri, an anti-Rh coat known to detect a majority of rheumatoid factors(23). That is, agglutination by serum F.S. of cells coated with anti-D Ri could not be inhibited with a number of different Gm(a—) sera. A weakly reactive latex fraction II fixation test was present in serum F.S., as has been seen in serum from non-rheumatoid patients with anti-Gm agglutinators resulting from multiple transfusions(2). Further studies on the interrelationships of such types of anti-γ globulin activities are in progress.

Antibodies found in human serum have in many instances been shown to reside in more than a single immunoglobulin class. Thus, anti-Rh(24), anti-ABO blood group (16), and antibacterial antibodies(25,26) have been described in γG, γM and γA immunoglobulins. Anti-Gm agglutinators of the non-rheumatoid type have been found most commonly among the γM globulins, but also occasionally as mercaptoethanol resistant antibodies with characteristics of γG globulins (2,4). The documentation of such activity in the γA immunoglobulins offers further substantiation of the concept that such activity is truly antibody in nature.

Summary. A serum is described in which anti-Gm(a) activity with characteristics of that found in non-rheumatoid patients was found to have certain unique properties. Most but not all activity was removed by reduction with 0.1 M β mercaptoethanol. Sephadex and DEAE cellulose chromatography excluded anti-Gm(a) activity from the 7S γG globulins. Studies with sucrose density gradients indicated major anti-Gm(a) activity in a position intermediate between the 19S and 7S immunoglobulins, with an S rate estimated to be between 11.0 and 11.8. Absorption studies showed selective removal of anti-Gm(a) activity from this serum by anti-γA antisera. This evidence supports the contention that anti-Gm activity resides in the γA globulins. The finding of such activity in multiple immunoglobulin classes is in keeping with the concept that anti-Gm agglutinators of this type are antibody in nature.

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Agglutination of the H-Viruses with Various Types of Red Blood Cells.* (31685)

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Although it has been noted that the H-viruses(1), including Kilham's RV(2), are able to agglutinate several types of red blood cells(3), no comprehensive data on this phenomenon has been reported. Recently, the discovery of a number of small DNA viruses similar to the H-group, such as the adenovirus-associated agents(4,5,6,7,), and Crawford's MVM(8), have focused considerable attention on these minute viruses. Type of hemagglutination (HA) has been used as one method of differentiation between the various small agents and between these viruses and other agents such as polyoma(8). In some instances where investigators have not been fully aware of the range of hemagglutination capability of the H-viruses, greater faith has been placed on HA or lack of HA with one or two types of red cells as a definite diagnostic aid, than is warranted. A detailed survey of the HA patterns of the H-viruses should be of value, therefore, in future work with these particular agents as they have consistent and individual HA patterns which are reproducible. Designation as a member of the H-virus group, *per se*, should, of course, be determined by pathogenicity studies in newborn hamsters, *i.e.*, death or production of a "mongoloid-type" deformity(1,2).

Materials and methods. Five H-viruses (H-1, HT, H-3, RV and HB) derived from distilled water filtrates of infected baby hamster litters(9) were employed for this study. (H-viruses prepared from infected tissue culture stocks have given similar re-

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