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# Antibody Formation in Dysgammaglobulinemia *

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Immunization of patients with type I dysgammaglobulinemia, in which a deficiency of γG- and γA- and an increase in γM-globulins exists, has usually resulted in production of γM antibodies (1–6). In most instances, however, the techniques employed for the measurement of antibody activity have been semiquantitative, and in some cases it has been difficult to distinguish the antibody activities produced by the patient from those contained in the γG given as treatment. Careful study of antibody formation in this syndrome seemed of interest because of the relationship between γM and γG antibody formation (7–10) and also because of the possibility that a unique opportunity would be provided to obtain evidence in man of whether immunological "memory" might be exhibited with γM antibodies. Studies in animals have suggested that γM antibody formation does not lead to persisting immunological memory (11, 12).

Antibody formation in a previously reported patient (5) with type I dysgammaglobulinemia was examined further by immunization with bacteriophage ØX 174 (ØX). Because ØX is highly antigenic and the assay for antibody is sensitive and quantitative (13, 14), this immunization system is advantageous for investigation of the immune response in patients with impaired antibody formation (15, 16).

## Methods

*Patient.* The patient, J. T., had a γM concentration of 1.8 g per 100 ml and γG and γA concentrations of less than 2 mg per 100 ml as determined by immunodiffusion analysis. The concentration of γD in the patient's serum [1] was less than 0.003 mg per ml (normal concentration, 0.02 to 0.05 mg per ml). Although γG was not identified by immunoelectrophoresis or by micro-Ouchterlony double diffusion of the patient's serum obtained before treatment with Red Cross γ-globulin, its presence was demonstrated by Preer double diffusion (18); trace amounts of this immunoglobulin were therefore present. During the current study, the patient was receiving Red Cross γ-globulin (γG) as treatment, 16 ml or 2.64 g every 6 weeks. From day 191 to day 321 (Table I), γG therapy was discontinued. The γG concentration in the patient's serum during this interval declined to less than 7 mg per 100 ml. One lot of γG used for therapy was tested for anti-ØX activity and none was demonstrated.

*Measurement of serum immunoglobulins.* During the period of study γG- and γM-globulins in the patient's serum were measured by single diffusion (Oudin) as described by Rubinstein (19) with antisera specific for human γG-, γA-, and γM-globulins (20).

*Antigen.* The preparation of the bacteriophage ØX has been described previously (15).

*Immunization.* The patient was immunized by intramuscular injection in the deltoid area of 0.1 ml of a phage preparation containing $10^{11}$ particles per ml. Blood was obtained by venipuncture at intervals after immunization.

*Antibody titrations.* The assay for antibody was carried out by the methods described by Adams (21).

*Physicochemical characterization of antibody.* Density gradient ultracentrifugation was performed with a Spinco model L ultracentrifuge and the SW-39L swinging-bucket rotor. Five-tenths ml of a 1:2 dilution of whole serum was layered on a gradient of 10 to 40% sucrose prepared by a density gradient sedimentation de-

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[1] Determined by Drs. David Rowe and John Fahey (17).

vice [2] and centrifugation carried out for approximately 16 hours. To ensure that material from the upper portions of the tube was not contaminated with material from the lower portions (12, 22), we removed 10 fractions by means of capillary pipettes from the top of the tube and 6 fractions through a perforation at the bottom of the tube. Marker proteins of known sedimentation coefficient were added to the sample to establish the approximate sedimentation coefficients of the various fractions. The marker proteins included aldolase, 8 S [3]; catalase, 11 S [4]; and rabbit thyroglobulin, 19 S.[5] Evans blue dye was added to identify the serum albumin, 4 S. Catalase was assayed as described by Chance (23 cf. 24). Aldolase was assayed as described by Allen, Sirisinha, and Vaughan (24). Thyroglobulin was detected by micro-Ouchterlony immunodiffusion analysis with a dog antithyroglobulin serum.[5] $\gamma$G, $\gamma$A, and $\gamma$M proteins were identified in micro-Ouchterlony immunodiffusion analysis with antisera specific for the corresponding immunoglobulins. The preparation of these antisera has been described by Leddy, Hill, Swisher, and Vaughan (20). The results shown in Figure 2 were obtained as indicated above, except that fractions of 0.24 ml were collected with the Isco density gradient fractionator.[6] IgM was quantitated by radial diffusion (25) with anti-$\gamma$M immuno-plates.[7]

Gel filtration on G-200 Sephadex was performed with a 0.8- × 113-cm column equilibrated in 1.0 M NaCl, 0.1 M pH 8.0 Tris-HCl. One ml of normal serum or the patient's serum was applied and eluted with the same buffer. One-ml fractions were collected, and their absorption at 277 m$\mu$ was determined. The fractions were analyzed for immunoglobulins by micro-Ouchterlony double diffusion employing antisera specific for $\gamma$G, $\gamma$A, and $\gamma$M.

Antibody was also treated with 2-mercaptoethanol (2-ME) as previously described (11). Antibody activity destroyed by 2-ME treatment was presumed to be 19 S, whereas that resistant to such treatment was presumed to be 7 S (26).

## Results

The results of immunization of the patient with ØX are shown in Table I. Serum obtained before immunization did not contain detectable anti-ØX activity. Fourteen days after immunization, the serum k (the rate constant of phage inactivation) for anti-ØX was 4.37, and all of this antibody activity behaved as 19 S. At 14 days, a second injection of $10^{10}$ ØX was given, and at 28 days the serum k was 14.3. Again all of the antibody activity behaved as 19 S. These results confirmed previous studies of the patient's immune response, in which only 19 S antibodies were produced (5). At 114 days, the serum antibody activity had declined to a total k of 0.575, but now antibody behaving in 2-ME as 7 S was detected. At 147 and 177 days, the serum k declined further, and by 177 days virtually all of the small residual antibody activity was of the 7 S type. At this time a third injection of $10^{10}$ ØX was given and 14 days later antibody activity again assayed. There were marked increases in both the 19 S and 7 S activities. Analysis of the 191-day serum by sucrose density gradient ultracentrifugation revealed the presence of slowly sedimenting and rapidly sedimenting antibody activities. As shown in Table II the slowly sedimenting antibody activity was resistant to 2-ME treatment, whereas the rapidly sedimenting antibody activity was largely destroyed by 2-ME treatment. By 307 days, the serum k value had declined to 0.64, with 0.5 as 7 S. After a fourth injection of ØX on day 307 marked in-

TABLE I

*Immunization of J. T. with bacteriophage ØX 174*

| Day | Dose of ØX* | Serum antibody (k)† 19 S | Serum antibody (k)† 7 S | Serum immunoglobulins $\gamma$G | Serum immunoglobulins $\gamma$M |
|---|---|---|---|---|---|
| | | | | *mg/100 ml* | |
| 0 | $1 \times 10^{10}$ | <0.01† | <0.01† | 94 | 1,210 |
| 14 | $1 \times 10^{10}$ | 4.37 | <0.01† | | |
| 28 | | 14.3 | <0.01† | | |
| 114 | | 0.187 | 0.388 | | |
| 147 | | 0.092 | 0.298 | | |
| 177 | $1 \times 10^{10}$ | 0.006 | 0.224 | 111 | 2,010 |
| 191 | | 23.2 | 2.21 | | |
| 307 | $1 \times 10^{10}$ | 0.14 | 0.5 | 188 | 1,695 |
| 321 | | 138.6 | 22.2 | | |
| 937 | $1 \times 10^{10}$ | <0.01‡ | 0.19 | 111 | 1,550 |
| 951 | | 320 | 240 | | |

* Number of plaque-forming particles injected.
† Here the identification of antibody activity as 7 S or 19 S was based on its susceptibility to 2-mercaptoethanol (2-ME). k is the rate constant of phage inactivation.
‡ Similar k with or without 2-ME.

TABLE II

*Anti-ØX 174 activity of 191-day serum*

| Antibody activity (k) | | | | | |
|---|---|---|---|---|---|
| Whole serum | | 19 S fraction* | | 7 S fraction* | |
| Without 2-ME | With 2-ME | Without 2-ME | With 2-ME | Without 2-ME | With 2-ME |
| 23.0 | 2.20 | 0.57 | 0.027 | 0.019 | 0.018 |

* Approximate, as determined by sucrose density gradient ultracentrifugation.

[2] Densigrad, Buchler Instruments, Fort Lee, N. J.
[3] Calbiochem, Los Angeles, Calif.
[4] Worthington Biochemical Corp., Freehold, N. J.
[5] Generously provided by Dr. Douglas Johnstone.
[6] Model D, Instrumentation Specialties Co., Lincoln, Neb.
[7] Lot 7022A6, Hyland Laboratories, Los Angeles, Calif.

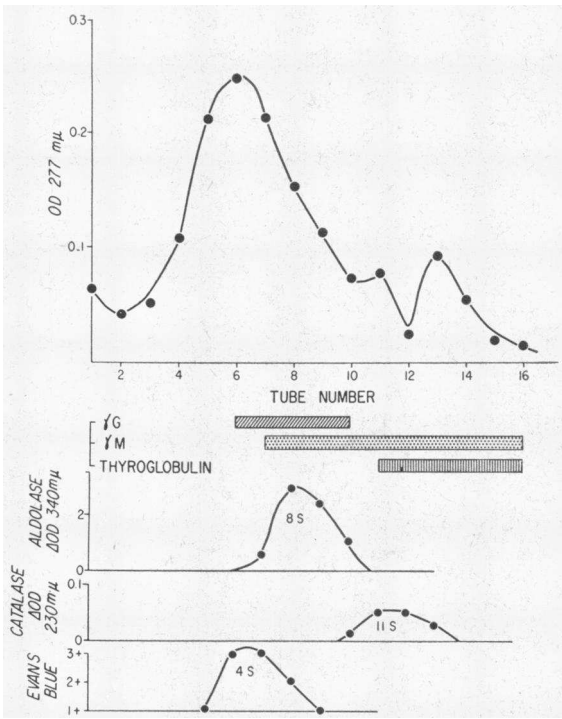


FIG. 1. SUCROSE DENSITY GRADIENT ULTRACENTRIFUGATION OF J. T.'S SERUM ON DAY 191.

creases in both 7 S and 19 S activities were noted; the 19 S antibody activity at this time was 31-fold greater than it had been after primary immunization (day 14). During the period from day 28 to day 177 the biological half-times, i.e., the resultant of synthesis and degradation, for the 19 S and 7 S antibody activities were 13 days and 80 days, respectively. Finally, on day 937 when 19 S antibody was not detectable, a fifth injection of ØX was given. There were striking increases in both the 19 S and 7 S antibody activities. The 19 S antibody activity was now 73-fold greater than after primary immunization.

The nature of the slowly sedimenting anti-ØX activity in the 191-day serum was examined further by sucrose density gradient ultracentrifugation and immunodiffusion analysis with antisera specific for γG, γA, and γM. These results are shown in Figure 1. Protein reacting with anti-γM was identified both in the 19 S fractions and also in the 7 S fractions. γG, passively acquired as treatment, was identified only in the 7 S fractions. As shown in Figure 2, gel filtration of the patient's serum on Sephadex G-200 also revealed protein reacting with anti-γM in the 7 S region. Noteworthy is the virtual absence of a 7 S peak in the patient's serum like that seen in the normal serum shown for contrast.

These studies indicate that the patient's serum contained slowly sedimenting (approximately 7 S) γM protein. This observation could have been caused by inadequate separation of 19 S γM from 7 S proteins because of the increased concentration of γM in the patient's serum. Density gradient ultracentrifugation of serum from a patient with macroglobulinemia showed, however, that even in the presence of a γM concentration of 3 g per 100 ml, 7 S γG- and 19 S γM-globulins could be adequately separated. Furthermore, when the fractions containing light γM from the patient were recentrifuged, the γM again sedimented in the 7 S area. Finally, as shown in Figure 3, repeat examination of the patient's serum by density gradient ultracentrifugation and analysis of the fractions for their concentrations of γM revealed two major

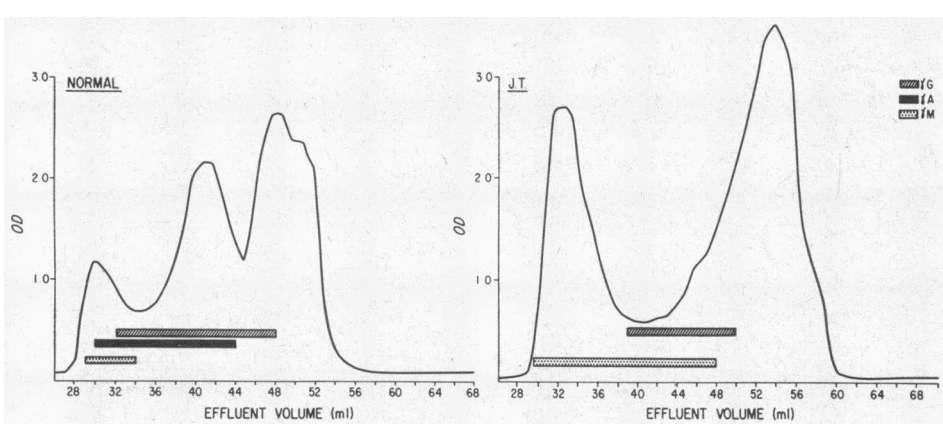


FIG. 2. GEL FILTRATION ON SEPHADEX G-200 OF J. T.'S SERUM OBTAINED ON DAY 191.

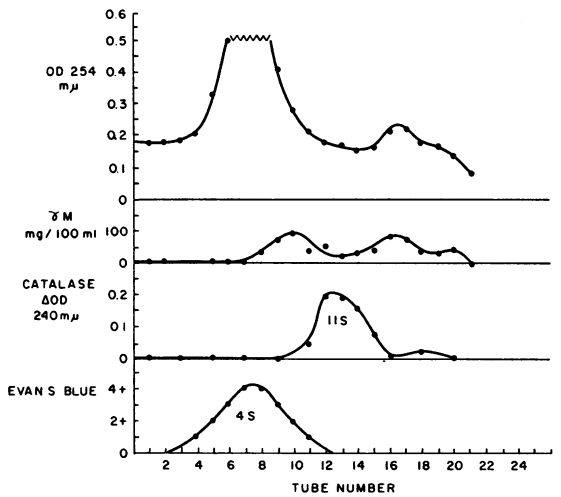


FIG. 3. REPEAT DENSITY GRADIENT ULTRACENTRIFUGATION OF J. T.'S SERUM.

peaks of γM protein, one faster and one slower than the 11 S catalase marker. These observations appear to exclude the possibility that the presence of the light γM was due simply to contamination of the 7 S area with 19 S γM-globulins and indicate that two populations of γM molecules were present in the patient's serum.

Since many of the sera employed in this study had been stored at − 10° C for periods up to several months, the possibility that the 19 S γM might have depolymerized to 7 S subunits was considered. Studies in our laboratory suggested that this may occur at least at 4° C, in the case of certain pathological macroglobulins (27). To examine this question, we subjected a sample of serum freshly taken from the patient to sucrose density gradient ultracentrifugation. The presence of γM was again demonstrated in fractions from the 7 S area. This observation demonstrates that the light γM was not related to prolonged storage of serum or to repeated freezing and thawing.

To determine whether the light γM possessed antibody activity, we examined its ability to neutralize ØX. Serum obtained on day 191 containing anti-ØX activity in the slowly sedimenting fractions was separated by density gradient ultracentrifugation, and the fractions from the 7 S area were pooled and dialyzed against saline. In these separations the patient's isoagglutinin, anti-B, served as a marker for the 19 S-containing fractions, and the therapeutically administered Red Cross γG-globulin served as a marker for the 7 S fractions. The 7 S pool was divided into 5 fractions and treated as shown in Table III. One fraction was absorbed with anti-γG, a second fraction with anti-γA, and a third fraction with anti-γM. To the fourth fraction, 13 μg N egg albumin was added, and this was absorbed with anti-egg albumin. This fraction served as a control for nonspecific effects of immunoprecipitation. A fifth sample served as a control to indicate the starting quantity of antibody. All of the antisera employed in these absorptions were shown by prior analysis to be free of anti-ØX activity. As indicated in Table III, absorption with anti-γG essentially completely removed the anti-ØX activity. Absorption with anti-γM and anti-γA reduced the activity by approximately 50%. These latter reductions may reflect the presence of specific antibody in the γA and the slowly sedimenting γM, or they may have been due to residual anti-γG activity in the form of γG-anti-γG complexes in the absorbed anti-γM and anti-γA sera. It appears, at any rate, that the 7 S neutralizing antibody was associated principally with the γG class of immunoglobulins; trace quantities of this class had been demonstrated in the patient's serum before any treatment with Red Cross γG-globulin. These separations and analyses were repeated on another occasion, and essentially the same results were obtained.

## Discussion

Immunization of patients having type I dysgammaglobulinemia (1–6) with antigens such as typhoid vaccine, tetanus toxoid, and poliomyelitis (Salk) vaccine has, in general, resulted in rather low levels of antibody activity. However, these

TABLE III

*Absorption of J. T.'s "7 S" fraction from density gradient ultracentrifugation*

| Antiserum used for absorption | k |
|---|---|
| Anti-γG | <0.0005 |
| Anti-γA | 0.0080 |
| Anti-γM | 0.0096 |
| Anti-EA* | 0.0150 |
| Control | 0.0170 |

* 13 μg N crystalline egg albumin (EA) added before absorption.

patients have often shown strikingly high titers of 19 S isoagglutinins against A or B erythrocytes, as was true also with our patient (5). Against typhoid, our patient produced low titers of 19 S anti-H and anti-O, and these titers remained low and as 19 S on reimmunization. The insensitivity and the semiquantitative nature of this agglutinin assay, however, prompted the present further investigation with ØX.

The antibody response to ØX in normal humans and other mammals consists of an initial short-lived formation of rapidly sedimenting (19 S) antibodies and a later longer-lasting formation of slowly sedimenting (7 S) antibodies (11). Immunological memory has been demonstrated for both 19 S and 7 S antibodies. However, the "memory" associated with 19 S antibody is usually transient; it was no longer demonstrable in guinea pigs when the interval between the first and second stimulation was 1 month, whereas that associated with 7 S was persistent for a minimum of 1 year. Similar results have been obtained in studies with poliovirus (12). In contrast to these results, Nossal, Ada, and Austin (28) have obtained 19 S immunological memory by challenging rats with minute amounts of flagella antigen followed 6 weeks later with a larger dose. Also, Borel, Fauconnet, and Miescher (29) obtained a 19 S anamnestic response in neonatal rabbits immunized with human red blood cells.

The immune response to ØX in the present patient differed from that in normal subjects in several respects. First, the predominance of 7 S antibody usually seen with prolonged immunization did not occur, and most of the antibody found was 19 S even after the fifth injection of antigen. Seven S antibody was produced only late in the course of immunization but then in increasing amounts. Most of the 7 S anti-ØX activity was associated with γG as demonstrated by its removal after absorption with a specific anti-γG serum. Secondly, the duration of synthesis of 19 S antibody appeared to be prolonged, as shown by the half-time of 13 days during the period from day 38 to day 177. The biological half-time of $\gamma_1$-macroglobulin in normal human subjects as determined by Barth, Wochner, Waldmann, and Fahey is 5.1 days (30). Finally, the increase in the level of 19 S antibody after the third injection of antigen was suggestive of slight immunological memory. This was demonstrated more strikingly after the fourth and fifth injections of antigen, when the level of 19 S antibody was 31- and 73-fold, respectively, greater than after primary immunization.

The existence of long-lived 19 S immunological memory in this patient may reflect an intrinsic abnormality of her 19 S antibody synthesizing mechanism. Another possibility is that our patient's immunological status was changing during the rather long period of study. That this may occur is shown by the observations of Rosen and his colleagues (1) on a patient (F. E.) with congenital agammaglobulinemia who inexplicably began to produce measurable amounts of γM protein. Repeated analysis of the immunoglobulins of our patient during the past 3 years (see Table I) has not shown any remarkable changes; the γM concentration has varied slightly, the γG concentration has varied in relation to the injections of exogenous γG, and γA has not been detected. It must be noted, however, that the amount of 7 S antibody formed in response to injection of ØX in this patient has steadily increased. This may be the result of repeated immunization but also could indicate a change in the patient's immunological status during the period of study. Finally, a third possibility is that the normal controls of 19 S antibody synthesis are not operative in the patient, and in their absence the full capability of the 19 S system is revealed. It has been shown by Finkelstein and Uhr (8) in guinea pigs that the production of 19 S antibody can be inhibited by passive administration of 7 S antibody. Similar results have been obtained in other immunization systems in rabbits (9) and in humans (10). Hence, it is possible that 7 S antibody persisting from the primary response may inhibit a potential secondary 19 S response. In patients with type I dysgammaglobulinemia the marked deficiency of γG- and γA-globulins may allow γM antibody production to occur largely free of the inhibitory effects of γG antibody. None of these three possibilities can be entirely excluded although on the basis of the available data one might prefer the last explanation.

Although the slowly sedimenting γM in this patient's serum appears to be a naturally occurring component among her γ-globulins, its relation to 19 S γM is not clear. Killander (31) obtained

suggestive evidence for the presence of low molecular weight γM, and Rothfield, Frangione, and Franklin have described a slowly sedimenting γM protein with antinuclear activity in the sera of patients with lupus erythematosus (32). A 7 S γM has also been demonstrated in the sera of horses immunized with bacteria (33). Recently Solomon and Kunkel have reported in abstract (34) a patient with a low molecular γM paraprotein. Several hypotheses have been advanced by Rothfield and associates (32) to explain the presence of light γM. The protein may represent a γM heavy chain or a subunit analogous to those produced by thiol reduction but not incorporated into the 19 S molecule. It may represent an *in vivo* or *in vitro* breakdown product of normal 19 S γM. It may be a normal component of human γ-globulin, such as a monomer of 19 S containing both H and L chains. At the present time, a choice between these alternatives is not possible. Absorption of the slowly sedimenting antibody activity with antisera specific for the various immunoglobulins indicated that most of the antibody activity was associated with γG. However, in view of the reductions after absorption with anti-γM and anti-γA, the presence of some activity associated with these immunoglobulins cannot be excluded. Finally, whether the immunoglobulins of this patient are analogous to the somewhat similar and intriguing immunoglobulins of the elasmobranch, *Mustelus canis* (smooth dogfish) (35), cannot be determined at this time.

## Summary

Antibody formation in type I dysgammaglobulinemia, a syndrome characterized by deficiency of γA- and γG- and elevation of γM-globulins, was studied by immunization with bacteriophage ØX 174. The bulk of the antibody formed was of the 19 S type even after five injections of antigen. However, after the second injection of antigen and thereafter significant amounts of γG antibody were produced.

The increase in the magnitude of the 19 S antibody response on repeated immunization indicates that the 19 S antibody-forming mechanism developed long-lasting immunological "memory." Previous failures to demonstrate on γM anamnestic response to viral antigens in guinea pigs and rabbits may be related to the inhibitory effects of γG antibody on γM antibody production.

A slowly sedimenting γM protein was demonstrated in the patient's serum by sucrose density gradient ultracentrifugation and gel filtration on G-200 Sephadex. The significance of the light γM in immunological responsiveness and its place in 19 S γM metabolism require further investigation.

## Acknowledgment

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