QUANTITATIVE STUDIES OF THE IMMUNOGLOBULIN SEQUENCE IN THE RESPONSE OF THE RABBIT TO A SOMATIC ANTIGEN*

By W. A. ALTEMEIER, III,‡ M.D., J. B. ROBBINS,§ M.D., AND R. T. SMITH, M.D.

(From the Department of Pediatrics, University of Florida, College of Medicine, Gainesville, Florida)

(Received for publication 28 April 1966)

The immune response of mammals to most soluble protein and viral antigens appears to differ in several respects from that induced by the somatic antigens of Gram-negative organisms (1–12). Though both classes of antigens elicit varying quantities of γM-antibody, much less γG-antibody has been reported to appear in the serum following lipopolysaccharide immunization. Several experiments had failed to demonstrate any γG-antibody to this antigen (1, 5–7). More recently, several reports have indicated that relatively low titers may be detected following repeated large doses of somatic antigen (8–11).

The sequence of production of γM- and γG-antibody has also been reported to differ in response to O antigen. γG-globulin activity is found in the serum within a few days after the first γM activity following administration of most antigens to mature animals (1–4). In contrast, immunization with the somatic antigen evokes γM-antibody production within a few days of injection, but γG-antibody activity does not seem to appear for a month or more (5–7, 10–12).

Lastly, the failure of animals to demonstrate higher levels of anti-O activity in whole serum following each of repeated immunizations has often been interpreted as indicating a lack of immunological anamnesis to the somatic antigen (1, 6, 11). Although the increasing γG-antibody activity induced by repeated immunization over prolonged periods has suggested that some form of immunological memory of this class of antigen exists (10), the characteristics of a secondary response observed with protein antigens have not been reported.

Recent data of Robbins et al. (13) showed that highly purified γM anti-*Salmonella typhimurium* O antibody had as much as 1000 times the agglutinating, bactericidal, and opsonizing activity of its γG-globulin counterpart when compared on a molar basis. In addition, the procedure used for isolating these two antibodies from whole

*Supported in part by the National Institute of Child Health and Human Development (HD-00384-08 and HD-0054-06) the American Heart Association (64-G-158), and the Stargaart Allergy Research Fund.

‡Special Trainee in Pediatrics, National Institute of Child Health and Human Development (TI-HD-54).

§Career Development Award (HD-22856-02), National Institute of Child Health and Human Development.

hyperimmune serum unexpectedly yielded much more γG- than γM-specific antibody. Thus it seemed possible that differences in the immune response to proteins and these O antigens could be explained on a basis of differential sensitivities of detection of the two classes of immunoglobulins in whole serum.

In the studies to be described, the sequence of anti-O antibodies in the serum after primary and secondary stimulation was examined by a specific immunoglobulin assay. The sensitivity of detection of both γG- and γM-agglutinins was increased by specific anti-γG- and -γM-globulin reagents. This technique, similar to that described by Coombs et al. for erythrocytes (14), and used previously for O titration (15–17), augments agglutinin titers as much as 256-fold. Estimations were made of the concentration of each immunoglobulin antibody in a single serum sample during the O antigen response by calculating from concurrent titrations of isolated and purified γM and γG anti-*Salmonella typhimurium* O antibodies of known concentrations.

The data show that the immunoglobulin response to O antigens has all the characteristics described as occurring after immunization with protein antigens, including rapid appearance of γG-antibody in large amount after initial immunization and a secondary-type response to reinjection. Further, this technique permitted indirect examination of certain qualitative changes of both types of antibody during the course of the immune response

Materials and Methods

Preparation of Antigen.—*Salmonella typhimurium* strain 7 (4, 5, 12:z) was inoculated from stock frozen brain heart infusion slants into brain heart broth. After incubation with shaking at 37°C for 8 hr the bacteria were washed twice with saline, heated to 100°C for 2 hr, and washed, in order, with saline, 95% acetone, and finally saline. The suspension was adjusted to 1×10^{10} organisms per cc by spectrophotometric absorption, using as a reference a bacterial suspension standardized with a particle counter. It was necessary to use the antigen within 3 days to insure the reproducibility of titrations.

Immunization.—Adult hybrid rabbits screened for low or undetectable agglutinin levels for the *S. typhimurium* somatic antigen were injected intravenously with specified amounts of this antigen in 1 ml of saline. Sequential bleedings on the indicated days following immunization were made by intracardiac puncture or from the ear artery. After incubation for 1 hr at 37°C and overnight at 4°C the serum was separated by centrifugation and stored in sterile tubes at 4°C.

Exposure to Reducing Agent.—2-Mercaptoethanol (2-ME) was used in a manner similar to that described by Chan and Deutsch (18). Briefly, dialysis of the sera at 4°C against 0.1 M 2-ME in pH 7 buffered saline for 24 hr was followed by dialysis against 0.02 M iodoacetamide for 6 to 8 hr, and then saline for 5 to 7 days. Prolonged saline dialysis of the reduced serum was necessary to insure reproducible titers, especially those following antiglobulin potentiation. Purified γG-antibody was included with each reduction procedure as a control; no loss of agglutinating activity occurred in saline or after antiglobulin potentiation. The corresponding purified γM anti-O control lost over 95% of its saline-agglutinating activity.

Antiglobulin Reagents.—Specific goat anti-rabbit γM- and γG-globulin reagents were prepared by immunizing goats with purified rabbit γM-globulin (13) or with the peptide Fc

derived from papain-digested pooled isolated rabbit γG-globulin (19) emulsified in Freund's adjuvant. The schedule of γM-globulin injection for immunization was similar to that used for the preparation of the anti-fragment Fc reagent (20). The anti-γM serum was absorbed with insoluble rabbit γG-globulin prepared by cross-linkage of this protein with bis-diazobenzidine (BDB) (21). In a similar manner, the anti-Fc, hereafter referred to as anti-γG, was absorbed with small increments of the soluble Fab peptide, followed by insoluble BDB-linked Fab fragment when the anti-γG formed a barely perceptible precipitate with the Fab peptide. After the specificities of both reagents were established (see below) they were concentrated by 37% ammonium sulfate precipitation. The antiglobulin preparations were diluted with buffered saline to their respective volumes before absorption, sterilized by filtration (Millipore Corp., Bedford, Massachusetts), and stored in 5 ml aliquots at −10°C.

Bacterial Agglutination.—The tube dilution titrations were done as described (13) except that 0.25 ml of antigen suspension containing 1.25×10^8 bacteria was added to each tube. After agglutination titers were recorded, the bacteria were washed twice with 1.0 ml aliquots of saline at 0°C. The tubes were then centrifuged at 2000 *g* for 20 min and the supernatant was discarded after each washing. After the second wash, the bacterial pellet was resuspended in 0.5 ml of a 1:1000 dilution of the respective antiglobulin reagent in saline. After the tubes were reincubated at 37°C for 1 hr and 4°C for 24 to 36 hr, the titers were again recorded. Each serum specimen was assayed at least twice, and in most cases three or more times on different days to minimize inherent errors in tube dilution techniques. The variation of titers was within ±1 tube with rare exceptions.

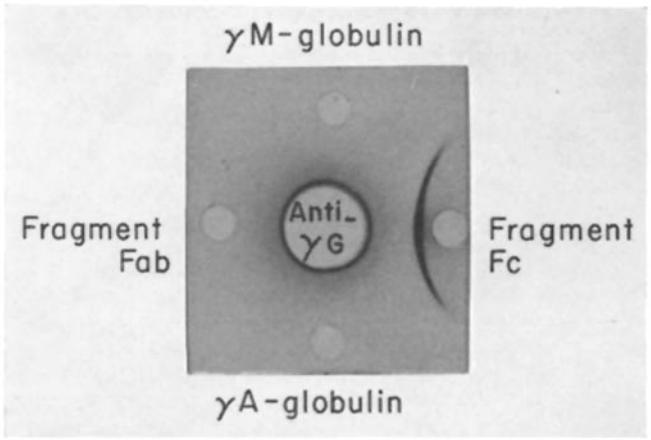
Controls and Standards.—Specified amounts of the isolated γM and γG anti-*S. typhimurium* antibodies mixed with 0.1 ml of normal rabbit serum containing no detectable agglutinins with or without antiglobulin augmentation were always included as a control for the agglutination and augmentation procedure. The purification and characterization of rabbit γG- and γM-antibodies for *S. typhimurium* somatic antigen has been described (13). Three different preparations of γM- and two preparations of γG-immunoglobulin antibodies were used in these experiments without significant variation in the specific agglutination activity. Immunoelectrophoresis, protein determinations, and sucrose gradient ultracentrifugation techniques, using standard procedures, were performed as described previously (13). Rabbit γA-immunoglobulin was prepared for immunodiffusion studies as described elsewhere (22).

Evaluation of Antiglobulin Augmentation Method.—Several procedures were employed in experiments to examine the reproducibility and specificity of the antiglobulin technique.

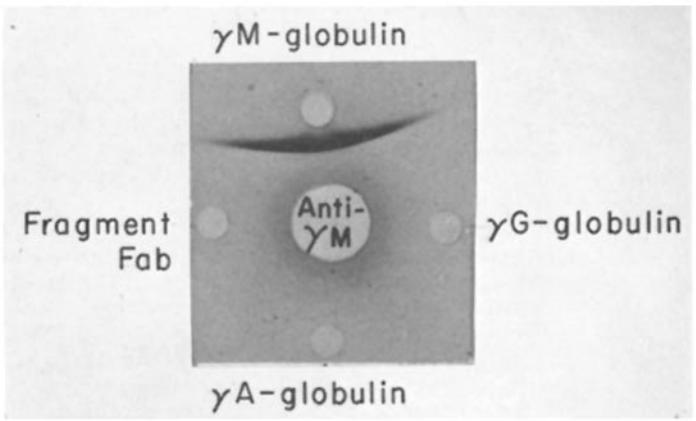
In agar diffusion analysis (Fig. 1 *a*, *b*) each goat antiserum would only precipitate its respective immunoglobulin. The antiglobulin reagents augmented only the titer of their respective isolated antibodies (Table I). The specificity of these reagents was also shown by the inability of the anti-γG-globulin to augment the titers of purified isolated γM and anti-*S. typhimurium* O antibody. In a similar fashion, anti-γM did not augment purified γG antibody-titers at the same concentration. Each antiglobulin would augment its corresponding antibody titer 8- to 16-fold at all dilutions tested. A dilution of 10^{-3} of each antiglobulin reagent, which yielded maximum enhancement of agglutinin activity, was chosen, and this concentration was used for all experiments.

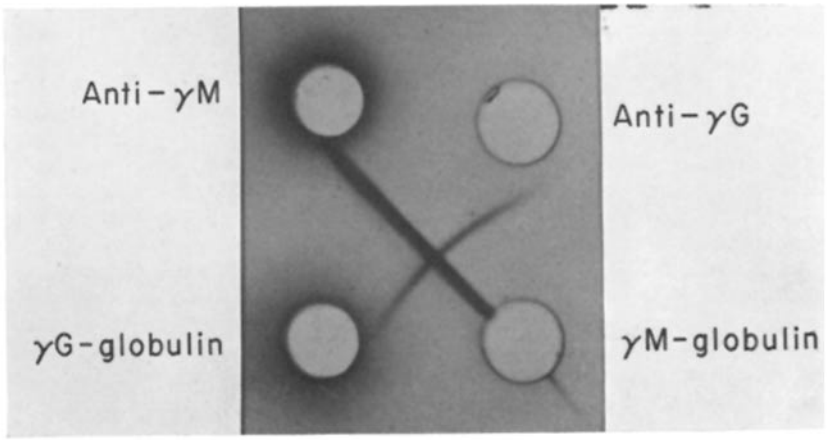
The specificity of the antiglobulin reagents in whole hyperimmune serum was also examined (Fig. 2). Analysis of the rapidly and slowly sedimenting fractions obtained by sucrose gradient ultracentrifugation revealed that goat anti-rabbit γM reagent increased titers associated with the rapidly sedimenting fraction and the anti-γG reagent augmented only the agglutinin activity of the more slowly sedimenting fractions.

Several possible sources of error may be introduced when the activity of one class of antibody is measured in the presence of the other in whole or 2-ME–treated serum. Since isolated γG and γM anti-*S. typhimurium* O appear to have specificity directed toward the same so-



1*a*





1*b*

FIG. 1. Immunodiffusion analysis of the goat anti-rabbit immunoglobulins, individually (Fig. 1 *a*) and in a "forced cross" pattern (Fig. 1 *b*). All antigen concentrations used were 1.0 mg/ml except for γA-globulin, which was 0.7 mg/ml. The anti-γG precipitated only fragment Fc of rabbit γG-globulin, and the anti-γM serum precipitated only γM.

matic antigen (13), mixtures of these in whole serum could conceivably be additive, yielding higher titers than either antibody would individually. On the other hand, γG-antibody with a relatively high avidity but low activity (13) could conceivably lower the measured titer of γM antibody by competing for antigenic sites. To assess these possibilities, isolated antibodies were combined in artificial mixtures which might simulate various conceivable conditions in whole serum. The results of representative experiments are given in Table II.

Concentrations of γG which were approximately twice those of the γM (Table II A) did not change the saline titer of the γM. The concentration of γG could be increased to as much as 17 times that of γM without changing the end point of the augmented γM titer (Table II B). Lastly, it was determined that reduced and alkylated γM-antibody in concentrations

TABLE I

*Selective Augmentation of Isolated γM and γG Anti-Salmonella typhimurium O Antibodies**

Antibody	Antibody concentration	Antiglobulin reagent dilution	Agglutinin titer			
			Saline	Anti-γM	Saline	Anti-γG
	mg/ml					
γM	0.88	1:100	320	5120	640	640
		1:500	320	2565	640	640
		1:1000	320	5120	640	640
		1:5000	320	2565	—	—
γG	0.57	1:100	20	10	—	—
		1:500	10	5	20	320
		1:1000	10	5	20	160
		1:5000	—	—	20	320

* Specific augmentation of agglutination activity of purified rabbit γM and γG anti-*S. typhimurium* O is shown. The titer is expressed as the reciprocal of the last dilution to show agglutination activity. The goat antiglobulin reagents were diluted to the indicated dilution and 0.5 ml of each preparation was added to bacteria coated with either antibody. The agglutination activity of each antibody was enhanced only with the specific anti-immunoglobulin reagent.

16 times higher than γG did not change the anti-γG augmented titers of the γG-antibody (Table II C).

Within the limits of these experimental variables, therefore, neither the saline nor the augmented titer of mixtures of the two antibody classes was significantly different from the highest titers of the separate antibodies. Therefore, γM-antibody activity was taken as the titer of whole serum before addition of goat anti-rabbit γM-globulin reagent, and the augmented titer was the titer after this addition. The activity of γG antibody was taken as the titer in reduced and alkylated serum diluted in saline, and the augmented titer was the titer after addition of anti-rabbit γG reagent to this serum. The expected minimum concentrations of antibody in whole serum detectable by this method would be: γM, 0.6 μg antibody protein per ml; γG, 3.3 γg antibody protein per ml (Table III).

EXPERIMENTAL RESULTS

The Primary and Secondary γM- and γG-Agglutinin Response to Salmonella typhimurium O Antigen.—In order to examine the relative titers of γM- and

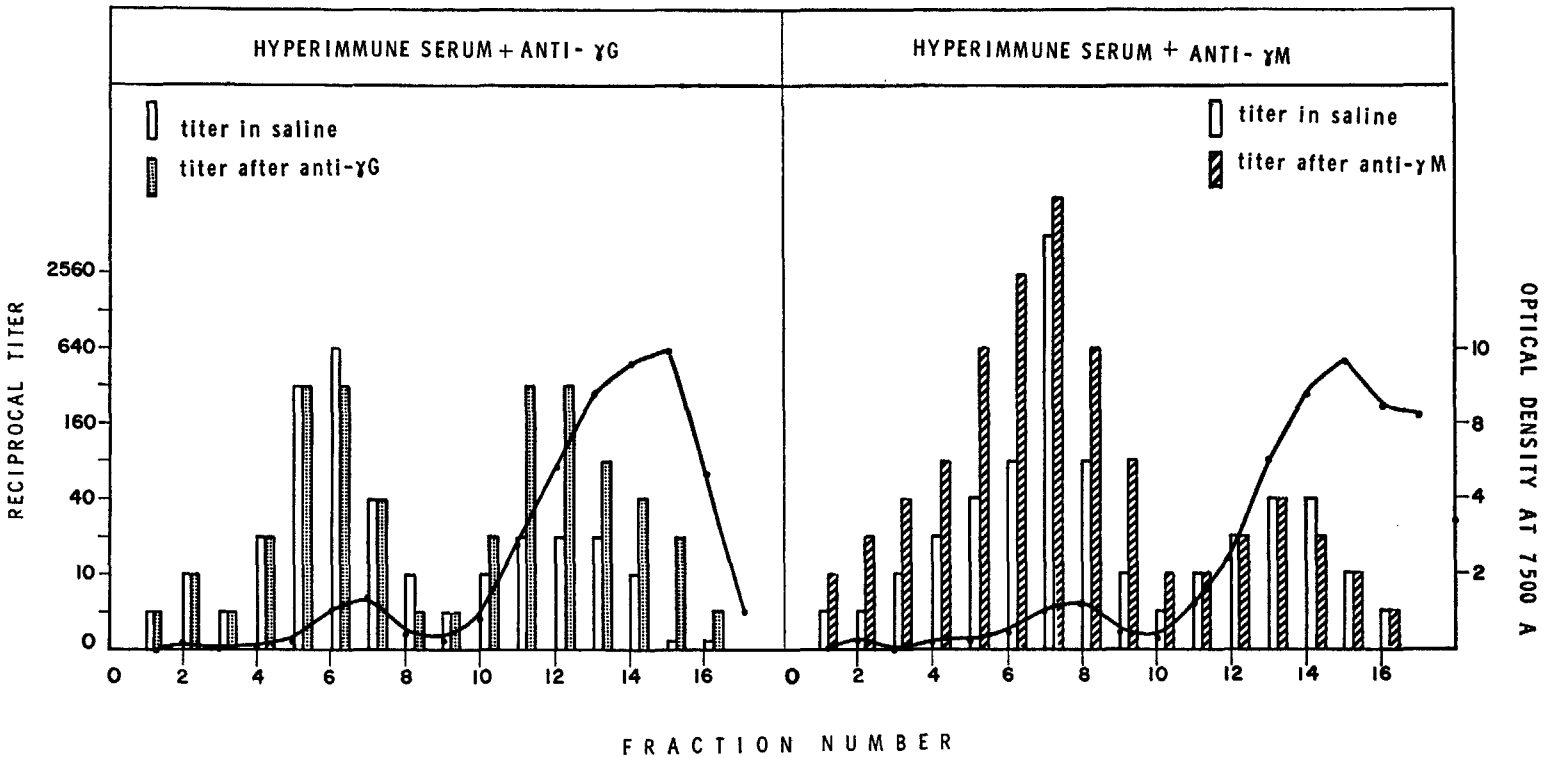


Fig. 2. Selective augmentation of γM and γG anti-O antibodies in whole hyperimmune serum subjected to sucrose gradient ultracentrifugation. Each fraction was assayed for agglutination activity in saline and after addition of the specific antiglobulin reagent. Adjacent bars represent titers before and after addition of the specific antiglobulin. The rapidly sedimenting agglutinins were augmented only by anti-γM, whereas the slowly sedimenting agglutinins were augmented only by the anti-γG reagent.

γG-antibodies in the primary and secondary response to the somatic antigen of *Salmonella typhimurium*, multiple serum samples from 10 immunized rabbits were examined by the direct and indirect techniques described. Groups of 3 or 4 rabbits received 1×10^8, 1×10^9, or 1×10^{10} organisms intravenously. After

TABLE II

*Agglutinin Activity of Various Mixtures of γM and γG Anti-Salmonella typhimurium O Immunoglobulins**

Experiment	Immunoglobulin in reaction mixture	Agglutination titer	
		Saline	Antiglobulin (reagent indicated)
	μg/ml		
A	1. γG (73.0)	5	—
	2. γM (38.0)	20	—
	3. γG (73.0) + γM (38.0)	20	—
B	1. γG (525)	40	20 (γM)
	2. γM (30.0)	10	160 (γM)
	3. γG (525) + γM (30.0)	40	160 (γM)
C	1. γM (330)	160	160 (γG)
	2. γM 2-ME (330)	0	0 (γG)
	3. γG (20.0)	0	10 (γG)
	4. γM 2-ME (330) + γG (20.0)	0	10 (γG)

* Mixtures of purified antibodies were prepared to simulate conditions in whole serum and assayed for agglutination activity in saline or after the addition of antiglobulin reagents. The titer is expressed as the reciprocal of the last dilution to show visible agglutination.

TABLE III

Concentration of Isolated Anti-O Antibodies Required to Agglutinate Salmonella typhimurium

Isolated antibody	Method of detection	Average minimum end point concentration of isolated purified antibody*	Minimum concentration of antibody detectable in whole serum‡
		μg/ml	*μg/ml*
γM	Titered in saline	2.5	13
	Anti-γM augmentation	0.121	0.6
γG	Titered in saline	16.5	83
	Anti-γG augmentation	0.66	3.3

* Each concentration represents an average of at least 50 titrations.

‡ This is the product of the average end point concentration (third column) and the first serum dilution (1.5) which could show agglutination in the standard titration method.

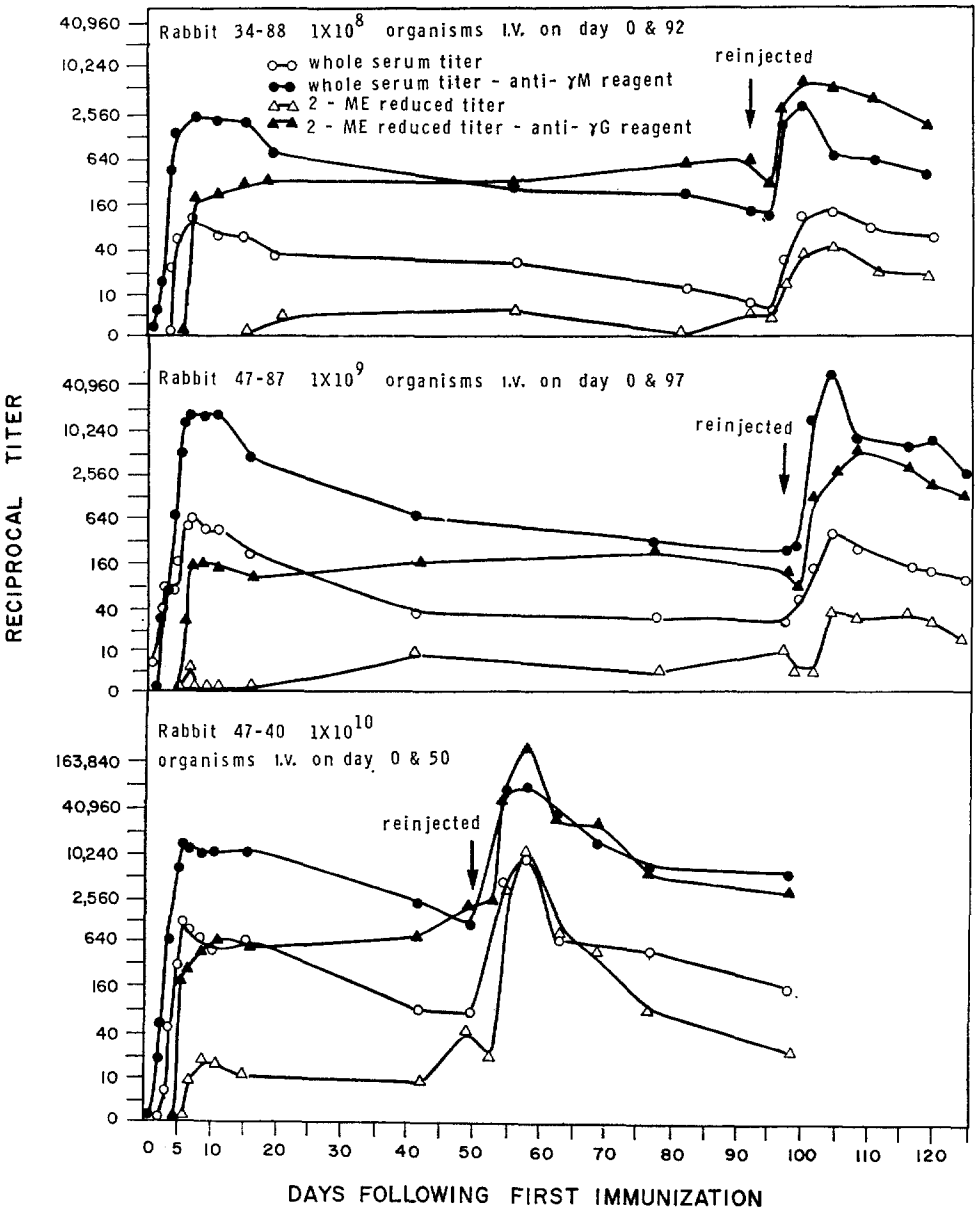


FIG. 3. The agglutinin activity of serial serum samples is illustrated for a representative animal from each group that received an intravenous injection of heat-killed *Salmonella tyhimurium*. The agglutination titer is recorded as the reciprocal of the last serum dilution to show agglutination.

immunization, the levels of agglutinating antibody were assayed at various intervals by the four techniques described. The titers of antibodies following initial immunization with the three doses of antigen are illustrated for representative animals in Fig. 3.

γM-agglutinins to the O antigen, as indicated by whole serum titers in saline or anti-γM augmented titers, appeared within 2 or 3 days after immunization. These agglutinin levels increased rapidly, reached a peak between 6 and 11 days, then fell gradually. γM-antibody activity was detectable even without antiglobulin augmentation as long as 126 days after exposure. These findings confirm prior data (1, 5–12).

Without augmentation, the titers of 2-ME–reduced serum in saline remained absent or very low during the immune response. These agglutinins usually appeared late in the response, although occasionally titers of 1:5 were detectable within a week of immunization. Even when saline γG-agglutinins were undetectable, γG-antibody was demonstrable by specific augmentation with the anti-γG reagent between 5 and 8 days after initial injection, except in a single animal in which γG first appeared at 17 days. Sucrose gradient ultracentrifugation of whole sera from such animals (Fig. 4) confirmed that these agglutinins were γG-immunoglobulins.

It was of interest to determine whether the early appearance of γG-agglutinins was related to the form in which O antigen was given. A lipopolysaccharide endotoxin of the same strain of *S. typhimurium* was purified by aqueous ether extraction as described by Ribi et al. (23). Rabbits were immunized with 2 or 20 μg of this preparation intravenously, and the serum collected at intervals during the subsequent 14 days was assayed by the technique described above. The γG- and γM-antibody response was similar in all respects to that observed following injection of the whole organisms (Fig. 3). γG-agglutinins were first detected by specific antiglobulin augmentation 6 days after immunization, and peak augmented titers of 1:1280 or higher were achieved by the 10th day.

The secondary response was studied by reinjecting 5 animals with the same dose of whole heat-killed organisms 50 to 126 days after initial immunization. The secondary response differed in several ways from the primary response (Fig. 3). In most animals, the maximum anti-O γM levels achieved after the second injection of antigen, as determined by both saline and antiglobulin titration, were comparable to the levels of γM anti-O detected after the primary response. The pattern shown by the animal illustrated in the lower section of Fig. 3 was the only exception to the observation that titers of agglutinins in whole serum were not significantly higher than those of the primary response. Thus, neither the earlier appearance nor the higher serum antibody titers characteristic of the secondary response were observed with these measurements of γM activity.

In contrast, the γG-agglutinin response to a second immunization appeared

earlier than the primary response. Augmentation with anti-γG revealed that the levels of γG-agglutinins were 10 to 100 times higher than those of the primary response in all but one animal. Thus γG-immunoglobulin agglutinins of the somatic antigen of *S. typhimurium* showed both of these characteristics

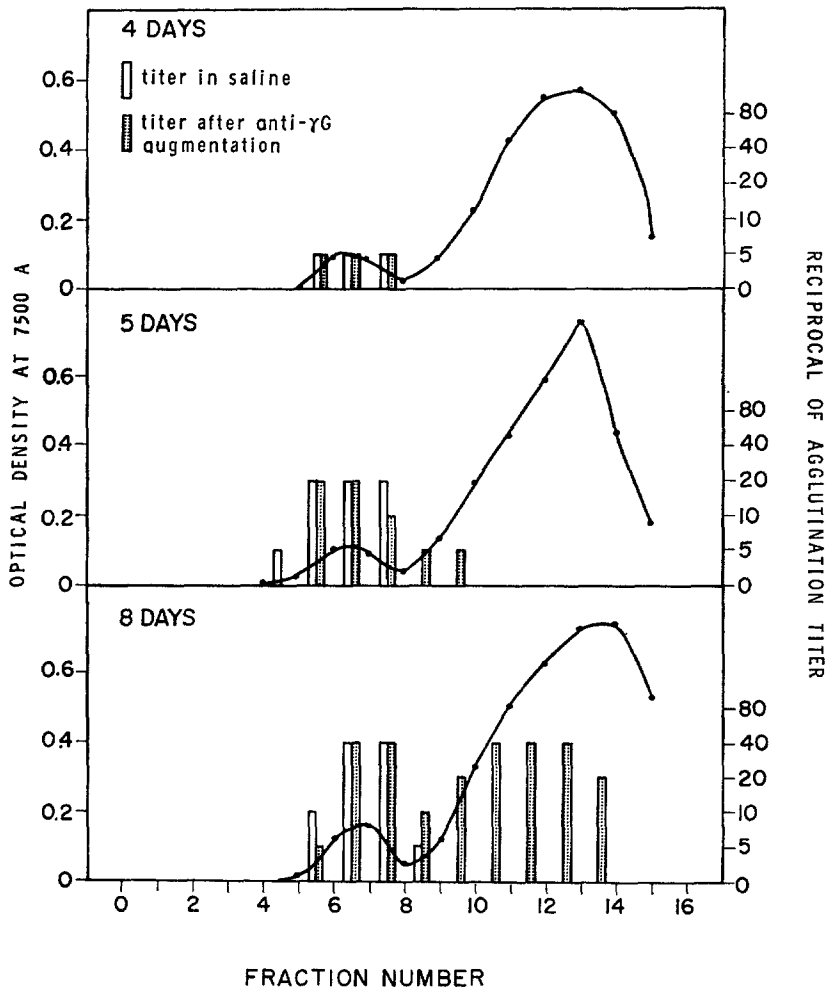


FIG. 4. Demonstration of γG-agglutinins by anti-γG augmentation of sucrose gradient fractions. Rabbit 34-88 received 1 × 10^8 *Salmonella typhimurium* on day 0. Sequential bleedings from day 4, day 5, and day 8 were examined for agglutinin activity in fractions of whole serum separated by sucrose gradient ultracentrifugation. Individual fractions were titered in saline (unshaded bar) and anti-γG (shaded bar). The curves represent the protein concentration in each fraction. By this technique specific anti-O γG antibody is detectable on the 5th day after immunization in whole serum samples.

of immunological anamnesis: earlier appearance and greater antibody production.

Estimation of the Relative Amounts of Immunoglobulin Antibodies Produced.— An objective of these experiments was to reexamine the sequence of formation of γM and γG anti-O agglutinins during the primary response. By increasing the sensitivity of detection with specific antiglobulins it was found that γG activity appeared in high titer within 3 to 5 days of γM-antibody rather than late in the response and at low levels. To conclude, however, that a 3 to 5 day interval separates the synthesis of the two classes of antibody is not necessarily justified. Such values represent only relative agglutinating capacity, not concentration of antibody.

A comparison of the actual amounts of each antibody necessary to cause agglutination and the sensitivity of detection of each in whole serum with the described titration techniques, given in Table III, shows that equal concentrations of γG- and γM-agglutinins yield different titers.

Isolated and purified γM and γG anti-*S. typhimurium* antibodies of known activity and protein content were used as standards to which titers in whole serum were compared in an attempt to estimate the concentration of the two antibodies. It was assumed that the isolated antibody gives a titration end point at the same concentration as the agglutinin being measured, and the product of the concentration of the standard agglutinin at its end point and the reciprocal of the titer of the unknown was taken as an estimate of the actual concentration of antibody in each unknown serum. Such estimates of antibody concentration give a more accurate picture of the timing of early antibody production, but they do not completely resolve sequence timing, for reasons that will be discussed.

The patterns of change in calculated concentrations of the two classes of antibodies during the primary response to *S. typhimurium* are shown for a representative rabbit in Fig. 5. The slope of initial rise of γG-antibody protein concentration usually paralleled that of γM and followed it by 2 or 3 days. In no animal studied was simultaneous appearance of the two immunoglobulins observed using these techniques.

Such calculations also indicate the accumulation of high concentrations of γM-antibody early in the primary response. The maximum levels were usually 1 to 3 mg of antibody protein per milliliter of serum and averaged 1.4 mg per milliliter. With few exceptions, however, the levels of γG-antibody ultimately equaled or surpassed the γM-antibody concentrations. These calculations compare γM- and γG-antibody production on a weight basis. Comparison of their molar concentrations (24, 25) does not change the basic shape of either curve, but results in an approximately 6-fold increase of the γG component. The initial slopes remain parallel, but the interval between the appearance of the two immunoglobulins is reduced to approximately 1.5 days.

Changes in Degree of Augmentation by Antiglobulin Reagents during the Primary and Secondary Response.—The quantitative estimations of immunoglobulin antibody concentrations described above are based upon the assumption that single isolated γM and γG standards are representative of the antibody produced in all phases of the primary or secondary response; i.e., that the γM- and γG-antibodies change quantitatively but not qualitatively during

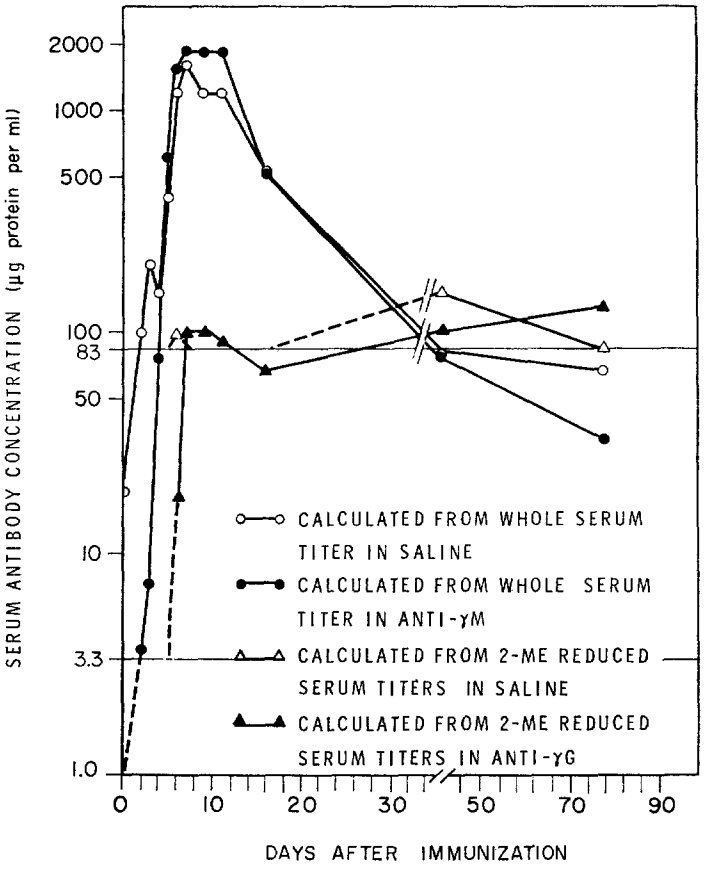


FIG. 5. Calculated levels of γM and γG anti-*Salmonella typhimurium* O antibodies during the primary response of rabbit 47–87 to 1×10^9 organisms given intravenously. Concentrations of antibody below the limits of detection are shown only by the broken lines leading to the first detectable antibody level. The horizontal lines represent the smallest serum concentrations of isolated γG detectable in saline (83 μg/ml) and after anti-γG augmentation (3.3 μg/ml). The early divergence in γM concentrations calculated from whole serum and augmented titers was more pronounced in animals having low preimmunization agglutinins. Much less difference was evident in the first calculated levels of γM from rabbits without preexisting agglutinins, such as 34–88 (Fig. 3).

this period. Variations in avidity characteristics during the primary and secondary responses in several other systems (26–32) suggest that this assumption is not necessarily valid.

A qualitative change in both γG- and γM-antibodies during the immune

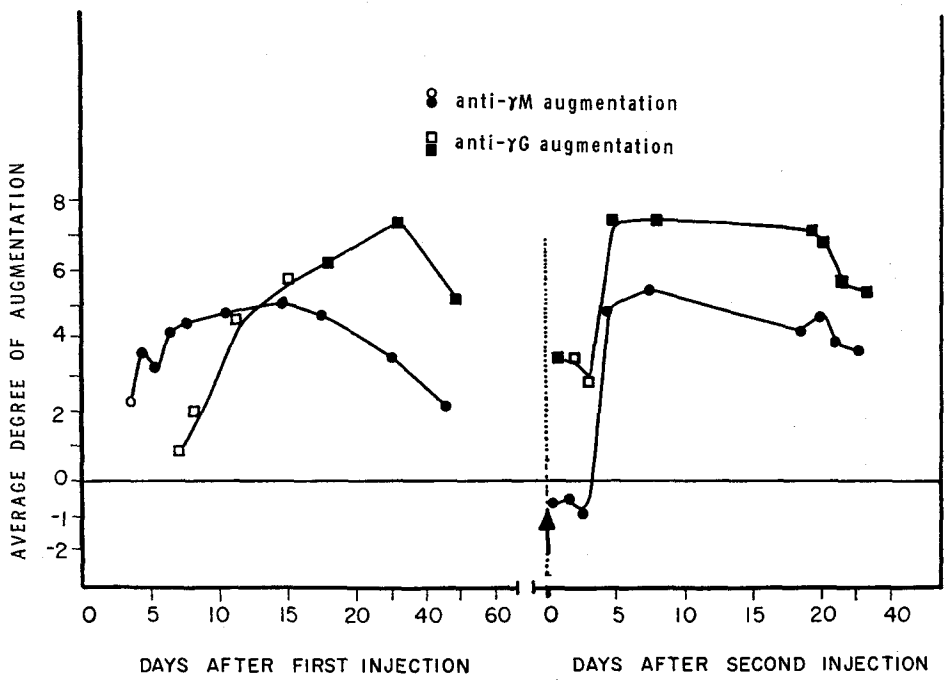


FIG. 6. Variations in the degree of augmentation during the course of γG and γM anti-O production by a representative rabbit (47–04) which received 1×10^8 organisms intravenously on days 0 and 126. The degree of augmentation is represented by the difference between the average number of tubes with agglutination after augmentation, and the average number of positive tubes in saline. For example, a value of +3 indicates that the titer was 3 tubes higher after augmentation, and represents an 8-fold increase in titer. In those cases in which augmentation could not be estimated because the titer in saline was less than 1:5, the degree of augmentation was calculated from an assumed base line of 1:5; in this case the circle or square is open.

response is indicated by the varying ability of antiglobulin reagents to augment the agglutinating activity of both immunoglobulins. This is illustrated in Fig. 6. A progressive increase in the degree of augmentation of whole serum titers of γM occurred during the first 2 weeks of the primary response. This effect was most marked in animals with low levels of preimmunization agglutinins, since in this case the earliest titers of serum samples often declined after the addition of the anti-γM reagent. The degree of γM-agglutinin augmenta-

tion increased, then fell slowly late in the primary response, and increased again after reimmunization. Similar analysis of the primary response γG augmentation was limited because 2-ME–resistant agglutinins in saline were frequently absent and thus provided no base line to determine the degree of augmentation. However, the same pattern was suggested in those few bleedings which had low levels of saline γG-agglutinins after primary immunization. Serial changes in degree of augmentation were clearly evident for γG-agglutinins during the secondary response.

These variations in augmentation obviously affect the accuracy of the quantitative estimates of antibody protein by the indirect methods employed here. The degree of augmentation of γM activity during the first 4 days of the immune response was always less than that of the isolated γM-antibody, or the γM activity in whole serum taken during the 2 weeks of peak γM production. Quantitative calculations must therefore be assumed inaccurate during the phase of the response when the first antibody is detected. Since the assay system is imprecise during the first days after antibody appears in the serum, it is difficult to be certain that γM-antibody actually appears before γG. γG levels early in the secondary response calculated from assay data are likewise misleading because of the higher degree of augmentation of whole serum γG-antibody as compared with the isolated γG-antibody.

DISCUSSION

The use of sensitive methods to measure agglutinating antibodies of the γG- and γM-globulin classes has been used to characterize the primary and secondary responses to somatic antigen in the form of whole heat-killed *Salmonella typhimurium*. In contrast to data reported previously (1, 5–12), γG-antibody usually appeared within 5 to 8 days of primary immunization and exhibited an initial increase in concentration roughly parallel to that of γM-antibody. After a second identical antigenic stimulus, γG-antibody activity began to increase sooner and reached titers 10 to 100 times those noted in the primary response, establishing a secondary response to this form of antigen.

The immune response of newborns to somatic antigens has been reported to be poor or absent in both human beings (33, 34) and rabbits (35). Reexamination of this response in newborn rabbits using antiglobulin augmentation in preliminary experiments indicates that low levels of γM- and γG-agglutinins of this O antigen are produced in a majority of animals. There remains little reason to consider the antibody response to somatic antigens to be different from that induced by protein and other antigens.

The quantitative techniques used gave peak γM levels that averaged 1.4 mg/ml. Such levels are high as compared with established values for total precipitating antibody, even after repeated immunization with the somatic antigen (9, 11). If these estimates are correct, it must be assumed that a large proportion of the agglutinating antibody does not precipitate with antigen.

Staub (36) found that agglutinin activity did not parallel precipitin content of serum from rabbits immunized with a purified somatic polysaccharide. Also, agglutinin activity was reduced but not abolished by removal with specific antigen of precipitins in immune rabbit serum. The very high antibody concentrations observed here perhaps explain the hypergammaglobulinemia reported to occur after intensive stimulation with somatic antigen (9, 12).

The timing of initial γM- and γG-antibody appearance in the serum during the primary response is a matter of considerable theoretical interest. It bears on the question whether a single cell differentiates through stages in which γM-antibody is produced followed by γG synthesis, or whether this event occurs in separate cell lines. This problem has in part been dealt with in the past by examining primary response sera for the earliest macroglobulin, and 7S immunoglobulin activities by a variety of methods. Since γM activity was usually found before that of γG, it appeared that a sequence was involved in their synthesis. Evidence has recently been presented that this may be due in part to greater sensitivity of γM-antibody detection in the systems used (13, 37, 38). With techniques that apparently detect lower levels of γG-antibody, it can be found sooner in the primary response (37, 38). Freeman and Stavitsky (39), using radioautographs, recently reported specific antigen binding by γG-globulins appearing simultaneously with γM-antibodies after primary immunization. However, the relative amount of each class of antibody protein measured by this technique is unknown. Therefore, unless equal sensitivity for both classes can be documented, inaccuracy in the timing of first appearance would be expected.

The problem is even more complex if the immunoglobulins undergo qualitative as well as quantitative changes during the interval when the sequence is to be examined, as appears to be the case from the studies we report here. In all experiments at least 1 or 2 days separated the first γM detected and the first γG-antibody appearing in serum. However, progressive changes in augmentation cast doubt upon this conclusion. This question can be approached more directly on a cellular level than by measurements of serum antibody. Studies using fluorescence microscopy to determine the sequence of γG- and γM-antibody–producing cells during a primary response are underway in this laboratory. Such experiments also have the advantage of avoiding added complexities of interpreting serum antibody measurements, such as differences in the vascular and extravascular distribution (40) and rates of catabolism of the two immunoglobulin classes (41).

The observed changes in degree of augmentation during various phases of the immune response remain unexplained, but best fit the concept that avidity is low early in the primary response and increases in the primary and early in the secondary response. If this is correct, the first antibody produced, being of relatively low avidity, would be eluted off the antigen during the washing process preceding addition of the antiglobulin reagent, and consequently slight

augmentation would be expected. The occurrence of changing degrees of avidity in itself requires further explanation in terms of theories of antibody formation and cell selection. One attractive possibility is that there is a feedback stimulation of those cells producing the more avid early antibody, resulting in the selection and stimulated proliferation of those ultimately producing the antibody of highest affinity for the specific antigenic determinant site. Data supporting the assumption of such a mechanism are unknown at present.

SUMMARY

Employing a sensitive and immunoglobulin-specific assay method based upon antiglobulin augmentation, quantitative and qualitative aspects of the primary and secondary response of the rabbit to *Salmonella typhimurium* O antigens have been evaluated. These studies examine the validity of the method of assay for detecting and measuring γG- and γM-antibodies produced in response to whole organisms or its lipopolysaccharide.

The results show that during the primary response γG-antibodies, not detectable by usual techniques, are produced in a pattern similar to that reported in animals stimulated by other classes of antigens. Moreover, the γG response following reinjection is characteristic of a secondary-type response. In contrast, γM-antibody levels after both primary and secondary stimulation rose equally to levels between 1 and 4 mg/ml. Despite increased sensitivity of detection and quantitative estimates of the actual molar concentration of each immunoglobulin, the minimal interval between γM and γG appearance in serum was not less than 1.5 days. The variable degree of augmentation of agglutination by antiglobulin reagent found during the immune response severely limits the quantitative usefulness of the methods developed. However, the data suggest that qualitative changes in anti-O antibodies interpretable as changes in avidity occur regularly during the immune response.

The authors are indebted to Mrs. Jean Curry for invaluable assistance.

BIBLIOGRAPHY

1. Bauer, D. C., Mathies, M. J., and Stavitsky, A. B., Sequences of synthesis of γ-1 macroglobulin and γ-2 globulin antibodies during primary and secondary responses to proteins, salmonella antigens, and phage, *J. Exp. Med.*, 1963, **117,** 889.
2. Uhr, J. W., and Finkelstein, M. S., Antibody formation. IV. Formation of rapidly and slowly sedimenting antibodies and immunological memory to bacteriophage ϕX 174, *J. Exp. Med.*, 1963, **117,** 457.
3. Svehag, S.-E., and Mandel, B., The formation and properties of poliovirus neutralizing antibody. I. 19S and 7S antibody formation: Differences in kinetics and antigen dose requirement for induction, *J. Exp. Med.*, 1964, **119,** 1.
4. Svehag, S.-E., and Mandel, B., The production and properties of poliovirus neutralizing antibody of rabbit origin, *Virology*, 1962, **18,** 508.
5. Bauer, D. C., and Stavitsky, A. B., On the different molecular forms of antibody

synthesized by rabbits during the early response to a single injection of protein and cellular antigens, *Proc. Nat. Acad. Sci.*, 1961, **47,** 1667.

6. LoSpalluto, J., Miller, W., Dorward, B., and Fink, C. W., The formation of macroglobulin antibodies. I. Studies on adult humans, *J. Clin. Invest.*, 1962, **41,** 1415.
7. Fink, C. W., Miller, W. E., Dorward, B., and LoSpalluto, J., The formation of macroglobulin antibodies. II. Studies on neonatal infants and older children, *J. Clin. Invest.*, 1962, **41,** 1422.
8. Pike, R. M., and Schulze, M. L., Production of 7s and 19s antibodies to the somatic antigens of *Salmonella Typhosa* in rabbits, *Proc. Soc. Exp. Biol. and Med.*, 1964, **115,** 829.
9. Weidanz, W. P., Jackson, A. L., and Landy, M., Some aspects of the antibody response of rabbits to immunization with enterobacterial somatic antigens, *Proc. Soc. Exp. Biol. and Med.*, 1964, **116,** 832.
10. Landy, M., Sanderson, R. P., and Jackson, A. L., Humoral and cellular aspects of the immune response to the somatic antigen of *Salmonella enteritidis*, *J. Exp. Med.*, 1965, **122,** 483.
11. Mulholland, J. M., Wolff, S. M., Jackson, A. L., and Landy, M., Quantitative studies of febrile tolerance and levels of specific antibody evoked by bacterial endotoxin, *J. Clin. Invest.*, 1965, **44,** 920.
12. Rowley, D., and Turner, K. J., Increase in macroglobulin antibodies of mouse and pig following injection of bacterial lipopolysaccharide, *Immunology*, 1964, **7,** 394.
13. Robbins, J. B., Kenny, K., and Suter, E., The isolation and biological activities of rabbit γM- and γG-anti-*Salmonella typhimurium* antibodies, *J. Exp. Med.*, 1965, **122,** 385.
14. Coombs, R. R. A., Mourant, A. E., and Race, R. R., Detection of weak and "incomplete" Rh agglutinins. A new test, *Lancet*, 1945, **249,** 15.
15. Morgan, W. T. J., and Schütze, H., Non-agglutinating antibody in human antisera to *Sh. shiga* and *S. typhi*, *Brit. J. Exp. Pathol.*, 1946, **17,** 286.
16. Stewart, F. S., and McKeever, J. D., The anti-globulin technique applied to the detection of non-agglutinating antibody against *Salmonella typhi* O in human sera, *J. Hyg.*, 1948, **46,** 357.
17. Turner, M. W., and Rowe, D. S., Characterization of human antibodies to *Salmonella typhi* by gel-filtration and antigenic analysis, *Immunology*, 1964, **7,** 639.
18. Chan, P. C. Y., and Deutsch, H. F., Immunochemical studies of human serum Rh agglutinins, *J. Immunol.*, 1960, **85,** 37.
19. Porter, R. R., The hydrolysis of rabbit γ-globulin and antibodies with crystalline papain, *Biochem. J.*, 1959, **73,** 119.
20. Jaquet, H., Bloom, B., and Cebra, J. J., The reductive dissociation of rabbit immune globulin in sodium dodecylsulfate, *J. Immunol.*, 1964, **92,** 991.
21. Bernier, G. M., and Cebra, J. J., Frequency distribution of, α γ, κ and λ polypeptide chains in human lymphoid tissues, *J. Immunol.*, 1965, **95,** 246.
22. Cebra, J. J., and Robbins, J. B., γ-A immunoglobulin from rabbit colostrum, *J. Immunol.*, 1966, in press.

23. Ribi, E., Haskins, W. T., Landy, M., and Milner, K. C., Preparation and host-reactive properties of endotoxin with low content of nitrogen and lipid, *J. Exp. Med.*, 1961, **114,** 647.
24. Lamm, M. E., and Small, P. A., Polypeptide chain structure of rabbit immunoglobulins. II. γM immunoglobulin, *Biochemistry*, 1966, **5,** 267.
25. Small, P. A., and Lamm, M. E., Polypeptide chain structure of rabbit immunoglobulins. I. γG immunoglobulin, *Biochemistry*, 1966, **5,** 259.
26. Jerne, N. K., A study of avidity based on rabbit skin responses to diphtheria toxin-antitoxin mixtures, *Acta Pathol. et Microbiol. Scand. Suppl.*, **87.**
27. Taliaferro, W. H., Taliaferro, L. B., and Pizza, A. K., Avidity and intercellular transfer of homolysin, *J. Infect. Diseases*, 1959, **197,** 105.
28. Grey, H. M., Studies on the binding between streptococcal γM protein and antibody, *J. Exp. Med.*, 1961, **115,** 671.
29. Grey, H. M., The quality of antibody produced by transferred cells, *Immunology*, 1962, **5,** 603.
30. Grey, H. M., Phylogeny of the immune response. Studies on some physical chemical and serologic characteristics of antibody produced in the turtle, *J. Immunol.*, 1963, **91,** 819.
31. Gilden, R. V., and Tokuda, S., Antibody quality after sequential immunization with related antigens, *Science*, 1963, **140,** 405.
32. Eisen, H. N., and Siskind, G. W., Variations in affinities of antibodies during the immune response, *Biochemistry*, 1964, **3,** 996.
33. Smith, R. T., Response to active immunization of human infants during the neonatal period. *Ciba Found. Symp. Cellular Aspects of Immunity*, 1960, 348.
34. Smith, R. T., and Eitzman, D. V., The development of the immune response. Characterization of the response of the human infant and adult to immunization with *Salmonella* vaccines, *Pediatrics*, 1964, **33,** 163.
35. Bellanti, J. A., Eitzman, D. V., Robbins, J. B., and Smith, R. T., The development of the immune response. Studies on the agglutinin response to *Salmonella* flagellar antigens in the newborn rabbit, *J. Exp. Med.*, 1963, **117,** 479.
36. Staub, A. M., Rôle des anticorps antipolyosidiques dans l'agglutination des bacilles typhiques, *Ann. Inst. Pasteur*, 1954, **86,** 618.
37. Benedict, A. A., Sensitivity of passive haemagglutination for assay of 7s and 19s antibodies in primary rabbit anti-bovine serum albumin sera, *Nature*, 1965, **206,** 1368.
38. Robbins, J. B., Altemeier, W. A., and Smith, R. T., The biological activity of rabbit anti *S. Typhimurium* immunoglobulins. An approach to the phylogenetic study of the development of immunity, *in* Phylogeny of Immunity, (R. T. Smith, editor), Gainesville, Fla., University of Florida Press, 1966, 76.
39. Freeman, M. J., and Stavitsky, A. B., Radioimmunoelectrophoretic study of rabbit anti protein antibodies during the primary response, *J. Immunol.*, 1965, **95,** 981.
40. Barth, W. F., Wochner, R. D., Waldmann, T. A., and Fahey, J. L., Metabolism of human gamma macroglobulins, *J. Clin. Invest.*, 1964, **43,** 1036.
41. Taliaferro, W. H., and Talmage, D. W., Antibodies in the rabbit with different rates of metabolic decay, *J. Infect. Diseases*, 1956, **99,** 21.