# Some Aspects of the Antibody Response of Rabbits to Immunization With Enterobacterial Somatic Antigens. (29386)

WILLIAM P. WEIDANZ,* ANNE L. JACKSON† AND MAURICE LANDY
*Laboratory of Immunology, NIAID, National Institutes of Health, and Department of Microbiology, Georgetown University School of Medicine, Washingtn, D. C.*

There is now a consensus that the immune response engendered by antigens involves production of at least 2 molecular species of specific antibody, and it has become increasingly apparent that the type of immunoglobulin synthesized by the antigen-stimulated host is dependent on many factors, including the physicochemical attributes of the antigen itself, quantity administered, route and schedule of injections and age and species of immunized host. Developments in this area are relatively recent, but the rapidly accumulating findings consistently show that the primary response to antigens such as sheep erythrocytes(1), bacteriophage(2,3), soluble proteins(4), bacterial flagellae(5), and poliovirus(6), is characterized by a transient rise in $\gamma$-1 macroglobulin antibody followed by the appearance of $\gamma$-2 globulin. Secondary response to these antigens results in production of predominantly $\gamma$-2 globulins. Little information is available on these aspects of the immune response to polysaccharide antigens, particularly the somatic components (endotoxin) of Gram-negative bacteria(7) which are now of special interest to the immunologist. The object of the present work was to determine the type of immunoglobulin produced by the rabbit in response to stimulation with these somatic antigens in the form of either intact Enterobacteriaceae or their isolated polysaccharides.

*Materials and methods. Antigens.* Saline suspensions of *S. enteritidis*, *E. coli* 0113 and *E. coli* 0127 were prepared from 18-hour cultures grown on brain-heart infusion agar, adjusted to approximately $1 \times 10^9$ cells/ml and heated for 2 hours at 100°C. Somatic antigen, extracted from *S. enteritidis* by the aqueous-ether procedure and further purified to remove most of the nitrogeneous moieties and much of the bound lipid(8), was provided by our colleague, Dr. Edgar Ribi; he also made available an isotopically labeled endotoxin prepared by cultivation of *S. enteritidis* in a synthetic medium in which uniformly labeled $C^{14}$ glucose was the sole carbon source. The purified somatic antigen extracted from these cells by the aforementioned procedure(8) had a specific activity of $10^4$ CPM/$\mu$g.

*Antisera.* New Zealand white rabbits, N.I.H strain, weighing 2-4 kg received intravenous injections of antigen in the form of heat-killed suspensions of *E. coli* 0127, 0113, or *S. enteritidis* 3 times weekly for a period of months. The initial dose was $1 \times 10^8$ cells; this was increased to $1 \times 10^9$ cells over a 2 week period, and this latter amount was continued thereafter. Other rabbits of the same strain received a similar course of intravenous injections of purified somatic antigen at an initial dose of 0.25 $\mu$g; this was progressively increased at weekly intervals to a maximum of 150 $\mu$g per injection. Animals were bled 4-6 days following the last injection and the sera were stored at —20°C. Most of these animals had been immunized to meet the needs of other investigations which required potent precipitating antisera. It is emphasized that the quantities of antigen and schedule of injections employed were empirical.

*Gel filtration.* Serum samples (1.0-1.5 ml) were applied to columns ($1.5 \times 100$ and $2.0 \times 100$ cm) of Sephadex G-200‡ and eluted in the cold at a rate of 4-9 ml/hr with veronal buffered saline (pH 7.4). Three to 5 ml fractions were collected by time or volume. Protein concentration was measured at 284 m$\mu$ and the fractions were stored at —20°C.

*Density gradient ultracentrifugation.* Sucrose density gradient centrifugation was per-

* NIH Postdoctoral Fellow—Present address: Louisiana State Univ., Baton Rouge.

† NIH Postdoctoral Trainee: Training Grant 5TIAI 182.

‡ Pharmacia Fine Chemicals, Inc., Rochester, Minn.

formed according to the techniques of Martin and Ames(9). Dialyzed serum (0.2 ml) diluted with an equal volume of phosphate (.05 M) buffered saline (pH 7.0) was applied to 4.8 ml of a 4-20% sucrose gradient and the surface layered with mineral oil. The tubes were centrifuged in a SW-39 swinging bucket rotor at 35,000 rpm for 13 hours in a Spinco model L centrifuge. Twenty-four serial drop fractions were collected from the bottom of each tube. One milliliter of 0.85% NaCl was added to each and the fractions were stored at —20°C.

*Bactericidal activity.* Antibody in the presence of complement is capable, under appropriate test conditions, of killing serum sensitive strains of *Enterobacteriaceae.* Precolostral calf serum was employed as a complement source free of antibody against the test strains of *S. typhosa* 0901 and *E. coli* 0127. Assay of the bactericidal activity(10) was performed by making suitable dilutions of the serum under test and adding an equal volume of calf complement. An inoculum of 100 viable cells was added and the mixture incubated at 37°C for 60 minutes followed by plating, overnight incubation and enumeration of the survivors. Antibody activity was expressed as the reciprocal of the serum dilution resulting in the killing of either 50% or 70% of the inoculum.

*Hemagglutinating antibody.* Hemagglutinating activity was determined by mixing 0.2 ml of 2-fold dilutions of serum or fractions with an equal volume of sheep erythrocytes previously coated with alkali-treated *S. enteritidis* endotoxin (10 μg antigen/ml 1% RBC). Agglutination was read after incubation for 2 hours at 37°C followed by 18 hours at 4°C. The titer was expressed as the reciprocal of the highest dilution of serum or serum fraction showing gross agglutination.

*Radioimmunoelectrophoresis*(11). Agar gel electrophoresis was performed with the sera of rabbits that had been given 28-70 injections of heat-killed *S. enteritidis.* Precipitin arcs of the serum proteins were developed by addition of goat anti-rabbit serum. The slides were washed and then flooded with biosynthetically labeled $C^{14}$ *S. enteritidis* endotoxin ($10^4$ cpm/μg); after 24 hours, the excess $C^{14}$ was removed by washing. Localization of specific combining antibody was determined by exposure of the slides to standard X-ray film for 38 hours.

*Results and discussion.* To ascertain the kinds of antibody produced by immunization of rabbits with a lengthy series of injections of heat-killed Gram-negative bacteria, immune serums were fractionated by gel-filtration on Sephadex G-200. Representative data for antisera against 3 enterobacterial species are given in Fig. 1, 2 and 3 as elution diagrams together with the antibody activities found in individual fractions. In Fig. 1 and 2 both the hemagglutination and bactericidal procedures disclosed the presence of specific antibody in the protein peaks associated with $\gamma$-$_1$ macroglobulin and $\gamma$-globulin(12). It is noteworthy that the rabbits given a total of $4.85 \times 10^{10}$ cells in 70 injections during a 5 month period (Fig. 2 and 3) developed a pronounced hypergammaglobulinemia; these serums had high levels of precipitating antibody (300-500 μg AbN per ml). However, the amount of hemagglutinating and bactericidal antibody under the 7S peak in no way paralleled the marked increase in $\gamma$-globulin. The distribution of antibody activities under the first 2 peaks differed considerably depending on the procedure employed for determination of antibody. Thus, in Fig. 2 most of the bactericidal activity (approximately 80%) was concentrated under the macroglobulin peak, whereas the preponderance of hemagglutinating activity was separable and distinct from the macroglobulin. The known differences in lytic and combining activity of 19 and 7S antibodies for red blood cells(1) probably account for these differences. The bactericidal assay appears to be much more responsive to 19S antibody whereas the hemagglutination procedure seems to favor the measurement of 7S over that of 19S antibody. The concentration of bactericidal activity under the first peak is all the more pronounced in view of the relatively small volume of effluent and the low concentration of protein present in this portion of the total serum. Moreover, in addition to the $\gamma_1$ macroglobulin, this peak

is also known to contain appreciable amounts of other non-antibody proteins such as $\alpha_2$ macroglobulin and lipoprotein(13), lending further emphasis to the marked biologic activity exerted by minute quantities of this specific protein.

Two of the aforementioned sera (Fig. 1 and 2) were also subjected to sucrose-density gradient ultracentrifugation and the individual fractions were tested for hemagglutinating activity. The findings (Fig. 4) affirm the results obtained by gel filtration in that this antibody activity was similarly distributed; it was present in both the fast and slow sedimenting fractions and the relative proportions were those found previously.

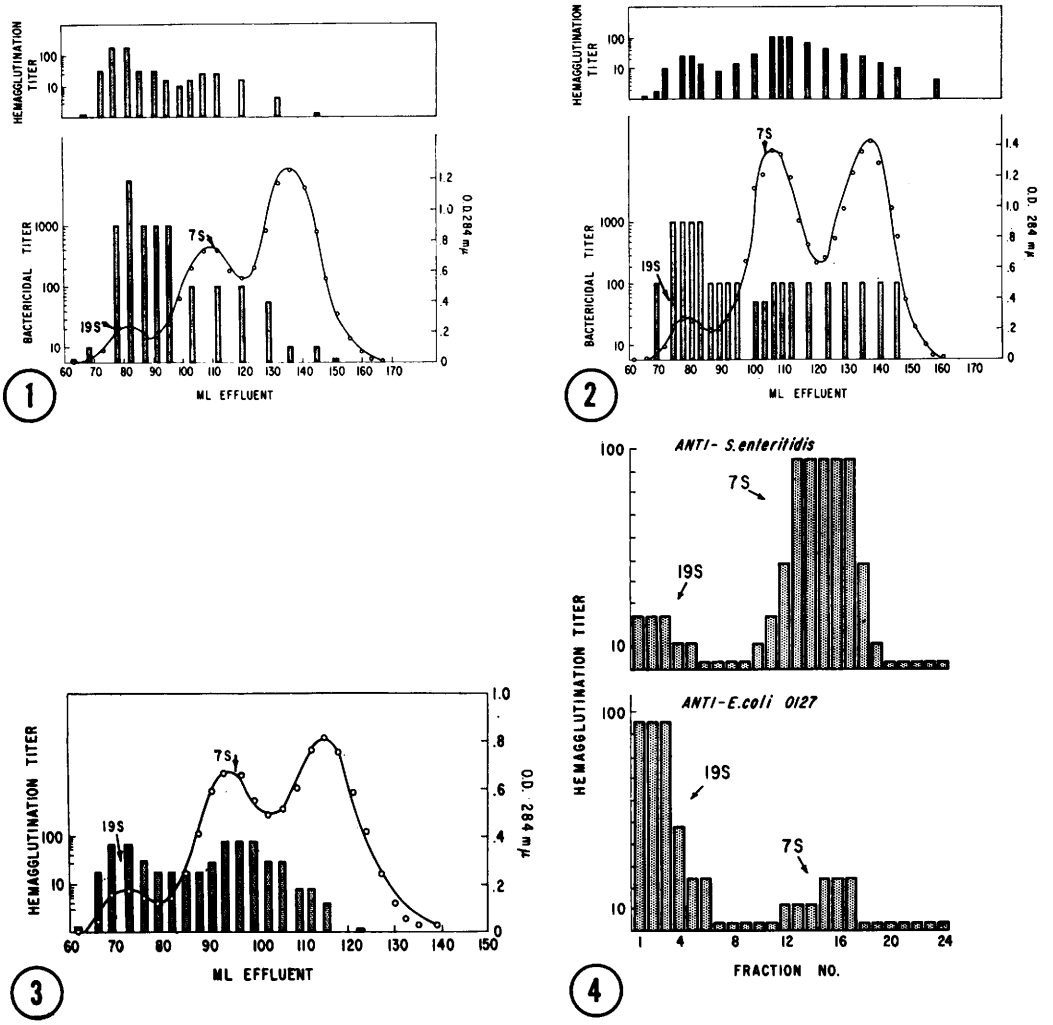


FIG. 1. Protein and antibody distribution following gel filtration of rabbit anti-*E. coli* 0127 serum. Immunization comprised 14 i.v. injections totaling 8.5 × $10^9$ heat-killed bacteria during a period of one month.

FIG. 2. Protein and antibody distribution following gel filtration of rabbit anti-*S. enteritidis* serum. Immunization comprised 70 injections totaling 4.85 × $10^{10}$ heat-killed bacteria over a period of 5 months.

FIG. 3. Protein and antibody distribution following gel filtration of rabbit anti-*E. coli* 0113 serum. Immunization schedule comprised 70 injections totaling 4.85 × $10^{10}$ heat-killed bacteria over a period of 5 months.

FIG. 4. Distribution of hemagglutinating activity obtained by sucrose density gradient ultracentrifugation of 2 rabbit antisera. Direction of sedimentation from right to left.

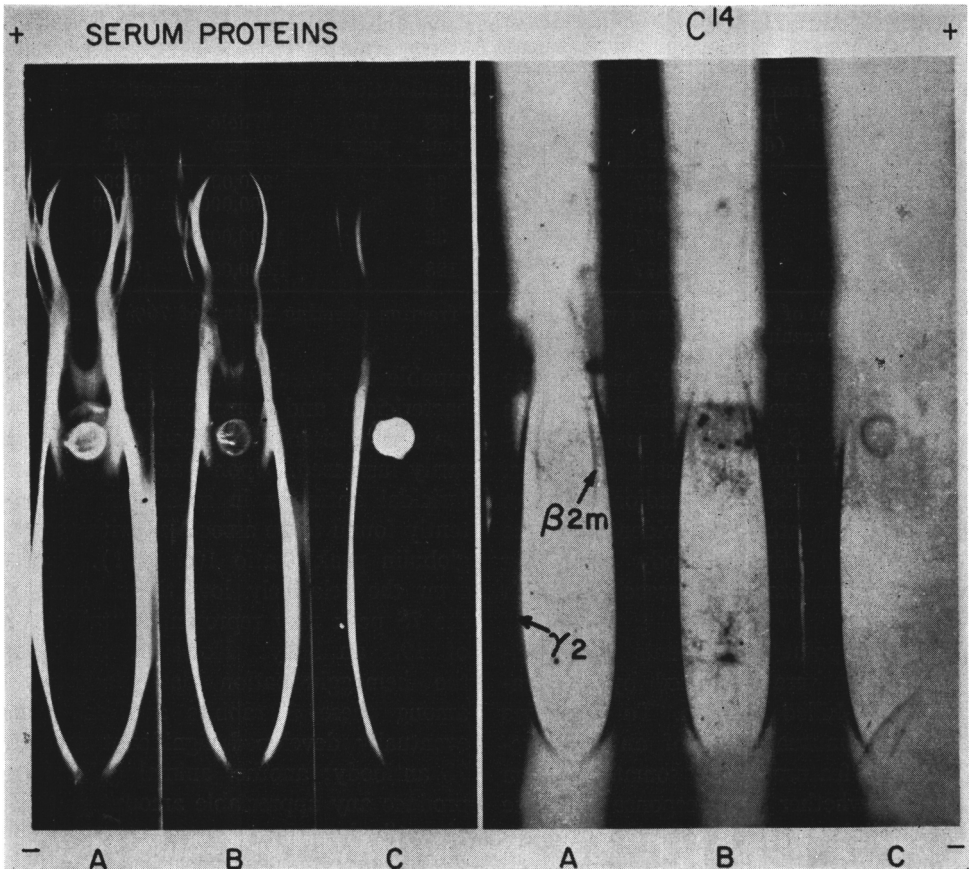


FIG. 5. Radioimmunoelectrophoresis of 3 rabbit anti-*S. enteritidis* sera. At left are individual precipitin arcs stained for protein with the corresponding autoradiographs of $C^{14}$ labeled endotoxin on the right.

These differences in antibody distribution could involve characteristics of individual antigen preparations, differences in immunization schedules, or possibly even reflect the immune responses of individual rabbits. Which of these factors constitutes the major influence on the type of immunoglobulin produced by the rabbit remains to be determined.

Employing the gel filtration technique used by Killander and Flodin(13) for separation of the saline type and incomplete type of blood group antibodies into high and low molecular weight serum components, it has been reported by us(14) (and by others(15), using different techniques) that in normal serum the bactericidal antibodies were restricted to the protein peak known to contain macroglobulins. The present findings extend our prior experience with gel filtration to immune sera and attest to the advantages and utility of this technique for separation and study of anti-"O" macroglobulin antibodies.

Radioimmunoelectrophoresis provided still another means of ascertaining which of the immunoglobulins in these hyperimmune sera contained specific activity. Duplicate serum specimens from rabbits A, B and C (immunized for 5 months with $4.85 \times 10^{10}$ cells of heat-killed *S. enteritidis*) were subjected to electrophoresis in gel. These tests were performed by Dr. H. C. Goodman and Mr. E. D. Exum of this laboratory, to whom we are greatly indebted. At the left side of Fig. 5 are shown the precipitin bands for the individual rabbit serum proteins as developed by reaction with goat anti-rabbit

TABLE I. Antibody Response of Rabbits Stimulated Intensively with Purified *S. enteritidis* Somatic Antigen.

| | Immunization | | | Hemagglutination titer | | | Bactericidal* titer | | |
|---|---|---|---|---|---|---|---|---|---|
| Rabbit | No. of inj | Time (days) | Antigen ($\mu$g) | Whole serum | 19S peak | 7S peak | Whole serum | 19S peak | 7S peak |
| I | 15 | 17 | 137 | 2,560 | 64 | 4 | 320,000 | 10,000 | 100 |
| | 27 | 58 | 1,477 | 2,560 | 16 | 16 | 100,000 | 1,000 | 10 |
| II | 27 | 58 | 1,477 | 1,280 | 32 | 8 | 100,000 | 1,000 | 10 |
| III | 27 | 58 | 1,477 | 1,280 | 128 | 4 | 1,000,000 | 10,000 | 100 |

* Reciprocal of the dilution of whole serum or fraction effecting killing of 70% or more of the bacterial inoculum.

serum. On the right side may be seen the radioautographs developed after interaction of the $C^{14}$ labeled *S. enteritidis* somatic antigen with the electrophoresed serum proteins. Although there is discernible radioactivity on the macroglobulin arc, it is evident that the preponderance of the antibody combining with the $C^{14}$ endotoxin is associated with 7S $\gamma$-globulin.

Up to this point experiments were concerned with antisera produced by stimulation with heat-killed bacteria. To determine whether this unusual type of antibody response was a feature of the somatic antigen as such, or whether this response was due to the cellular nature of the antigen (*i.e.* its situation on the bacterial cell wall) analogous experiments were carried out on the sera of rabbits injected with isolated somatic polysaccharides. As would be expected from prior work(16,17), even a single injection of 1 $\mu$g, or less, of *S. enteritidis* somatic antigen sufficed to evoke substantial levels of bactericidal and hemagglutinating activity. For example, 0.2 $\mu$g resulted in a bactericidal titer of 1:32,000; 4 additional injections (total of 1 $\mu$g) increased the titer to the range of 100,000-300,000. A single injection of 5 $\mu$g produced antibody levels in the latter range. Examples of antibody responses to immunization with much larger amounts of this antigen, as an extended series of injections over a longer time interval, are given in Table I. Included are antibody titers of the Sephadex G-200 fractions with the highest optical density at 284 m$\mu$ in the first 2 protein peaks. Although the total dosage of 1,477 $\mu$g represents more than 1,000 times the amount of this antigen capable of inducing relatively high levels of bactericidal and hemagglutinating antibody, the serum titers developed were not necessarily increased. Upon fractionation the bactericidal antibody in such sera was consistently found to be associated with the macroglobulin peak (ratio 100 to 1). Moreover, even the relatively low titers found under the 7S peak may represent a "tailing" effect of 19S antibody. However, inspection of the hemagglutination data indicates that among these 3 rabbits at least one (I) eventually developed significant amounts of 7S antibody; another animal (III) failed to produce any appreciable amount of this kind of antibody.

Previous work has been in agreement that the type of antibody produced following stimulation with somatic polysaccharides was exclusively macroglobulin in nature(2,18). The present work provides clear evidence that under unusual circumstances rabbits can also produce 7S antibody specific for these polysaccharide complexes. Although not clearly defined, it appears that repetitive stimuli and/or massive amounts of antigen in the form of bacteria are required to evoke significant amounts of 7S antibody. Even when obtained, this antibody was found to constitute by far the lesser of the 2 forms produced. However, it remains to be determined whether other conditions of antigenic stimulation, or whether other methods of measurement will disclose antibody formation more in keeping with the now well-established pattern(6) of transition from 19S to 7S immunoglobulin.

These experiments provide only a glimpse of the overall immunological response of the

rabbit to the somatic polysaccharides of Gram-negative bacteria. Further systematic work will be required to delineate the salient features of the hosts' immunological reactions to this kind of bacterial antigen. Nevertheless these experiments provide strong indications that the antibody response evoked by these polysaccharide complexes has distinctive characteristics which set it apart from the pattern established for other kinds of antigens.

*Summary.* The immunoglobulins of rabbits injected with bacterial cells or isolated somatic antigen of *Salmonella enteritidis* and *Escherichia coli* were separated by Sephadex G-200 gel filtration and the distribution of high and low molecular weight antibodies measured by bactericidal and other techniques. High molecular weight bactericidal antibody was the predominant form produced regardless of amount, form, or schedule in which somatic antigen was administered. However, evidence was obtained that significant amounts of 7S antibody could also be produced.

*Addendum.* The report by Pike and Schulze on production of 7S and 19S antibodies to the somatic antigen of *S. typhosa* in rabbits (Proc. Soc. Exp. Biol. Med., 1964, v115, 829) appeared after our manuscript had been submitted for publication. Although dealing with the same general issue these papers differ considerably; viz., the present work employed additional enterobacterial specificities, compared bacteria and isolated somatic antigens, utilized a different procedure for serum fractionation and depended on different methods for measurement of antibody activities.

1. Stelos, P., Talmage, D. W., *J. Infect. Dis.*, 1957, v100, 125.
2. Bauer, D. C., Stavitsky, A. B., *Proc. Nat. Acad. Sci.*, 1961, v47, 1667.
3. Uhr, J. W., Finkelstein, M. S., Baumann, J. B., *J. Exp. Med.*, 1962, v115, 655.
4. Benedict, A. A., Brown, R. J., Ayengar, R., *ibid.*, 1962, v115, 195.
5. Bellanti, J. A., Eitzmann, D. V., Robbins, J. B., Smith, R. T., *ibid.*, 1963, v117, 479.
6. Svehag, S. E., Mandel, B., *ibid.*, 1964, v119, 1.
7. Bauer, D. C., Mathies, M. J., Stavitsky, A. B., *ibid.*, 1963, v117, 889.
8. Ribi, E., Haskins, W. T., Landy, M., Milner, K. C., *ibid.*, 1961, v114, 647.
9. Martin, R. G., Ames, B. N., *J. Biol. Chem.*, 1961, v236, 1372.
10. Weidanz, W. P., Landy, M., PROC. SOC. EXP. BIOL. AND MED., 1963, v113, 861.
11. Yagi, Y., Maier, P., Pressman, D., *J. Immunol.*, 1962, v89, 736.
12. Killander, J., Flodin, P., *Vox Sanguinis*, 1962, v7, 113.
13. Flodin, P., Killander, J., *Biochem. Biophys. Acta*, 1962, v63, 403.
14. Landy, M., Weidanz, W. P., *Bacterial Endotoxins*, M. Landy & W. Braun, eds., Rutgers Univ. Press, New Brunswick, 1964.
15. Michael, J. G., Rosen, F. S., *J. Exp. Med.*, 1963, v118, 619.
16. Landy, M., Johnson, A. G., Webster, M. E., Sagin, J. F., *J. Immunol.*, 1955, v74, 466.
17. Landy, M., Trapani, R. J., Shear, M. J., *J. Exp. Med.*, 1959, v110, 731.
18. Lospalluto, J., Miller, W. E., Dorward, R., Fink, C. W., *J. Clin. Invest.*, 1962, v41, 1415.

Received April 22, 1964. P.S.E.B.M., 1964, v116.

## Suppression of Corticosteroid Production in the Dog by Monase. (29387)

WILLIAM W. TULLNER AND ROY HERTZ
*Endocrinology Branch, National Cancer Institute, National Institutes of Health, Bethesda, Md.*

Clinical toxicity encountered following administration of 3-(2-aminobutyl)-indole acetate (Monase*—Fig. 1) has included syncope and hypotension. Hence the effects of this compound on adrenocortical function were studied.

Pharmacologic agents which inhibit the secretion of adrenocortical hormones may act

* The Upjohn Co., Kalamazoo, Mich.