THE DEVELOPMENT OF THE IMMUNE RESPONSE

Studies on the Agglutinin Response to Salmonella Flagellar Antigens in the Newborn Rabbit*

By JOSEPH A. BELLANTI,‡ M.D., DONALD V. EITZMAN,§ M.D., JOHN B. ROBBINS,‡ M.D., and RICHARD T. SMITH, M.D.

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

Recently, the synthesis by human infants of a macroglobulin antibody to *Salmonella* flagellar antigens has been described, and a preliminary report of a similar phenomenon in the rabbit was made (1–3). In order to delineate the ontogenetic significance of macroglobulin antibody formation in the young mammal, the response to these antigens has been investigated further in the rabbit. The data described here delineate some of the variables of this response and indicate that the newborn rabbit develops a capacity for producing at least two components of the immune response, 19S gamma-1 macroglobulin and 7S gamma-2 globulin, in the same chronological sequence as the human infant.

Materials and Methods

Newborn rabbits were of hybrid New Zealand strains obtained by either purchase of pregnant does or by breeding in the laboratory. The does were allowed to nest in special breeding cages, where they remained with their litter until after weaning. Newborn rabbits were bled initially and their sera assayed for the presence of agglutinins to the flagellar antigens of *Salmonella paratyphi* B (1, 4, 5, 12:b) or *Salmonella sp.* (13, 23, 36:z) given hereafter as *S. 13, 23, 36: z*. Many litters were observed in preliminary studies in which the does—having had no known exposure to this group of organisms—had agglutinins in their sera. *S. paratyphi* B and *S. 13, 23, 36: z* agglutinins were least frequently encountered, therefore, in all experiments reported here these strains were employed. Only those animals found to have no agglutinins in a 1–5 dilution of prebleeding serum were used in these experiments.

Adult rabbits were similarly prebled and screened for pre-existing agglutinins, then received either one or two intradermal injections of 0.5 ml of the appropriate vaccine into the foot pad. In some experiments newborn rabbits received a series of three intraperitoneal or intradermal injections on alternate days with or without a final injection in the third week of life. In other experiments, a single intraperitoneal injection of 1.0 ml of the antigen was made on the first day of life.

The antigens used for immunization and agglutination tests were suspensions of highly motile organisms, grown on Difco tryptose blood agar base in Blake bottles. The organisms were harvested in 0.15 M NaCl, then killed with 0.6 per cent formalin in 0.15 M NaCl for 24 hours, and finally suspended in 0.15 M NaCl containing 0.05 per cent phenol as a preservative. The final concentration for most experiments was adjusted to approximately 10^9 organisms per ml by optical density measurement comparison with directly counted standards. For later experiments in which the number of organisms employed was the critical parameter, a Coulter counter was employed to determine the number to within 0.05 per cent.

The antigen used for detecting the somatic agglutinin was made by boiling the motile suspension for 1 hour and washing before use. These organisms were not agglutinated by appropriately absorbed control antisera against the monotypic phase I flagellar antigens. The organisms and certain control antisera were generously supplied by Dr. Phillip Edwards of the Enteric Diagnostic Unit of the Communicable Disease Center, United States Public Health Service, Atlanta.

Blood specimens were allowed to clot at room temperature; the serum was separated, rapidly frozen, and stored in sealed tubes at −20°C until used. Sera were heated 30 minutes at 56°C prior to determination of agglutinating antibody titer. The titrations were performed by adding a 1–10 dilution of the stock bacterial suspension to 0.25 ml of serial 1–2 dilutions of the serum in 0.15 M NaCl. After 1 hour at 37°C, and overnight at 4°C, final readings were made. Titers are expressed as the reciprocal of the highest dilution in which clear cut agglutination was easily visible to the unaided eye. No attempt was made to read partial agglutination, although it was evident in some determinations.

Starch block electrophoresis of 1.0 ml serum specimens was performed at 4°C, in pH 8.6, ionic strength 0.1 M barbital buffer using a 1 × 4 × 44 cm trough at 300 volts for 18 hours, a modification of the method of Kunkel and Slater (4). 1 cm cuts were eluted with 4.0 ml of 0.15 M NaCl, and the protein content estimated by the Folin-Ciocalteu technique (5). Flagellar agglutinins were detected in each fraction by titrating the eluates as described above.

Ultracentrifugal separation of the agglutinin activity of various sera was performed using the model E or model L Spinco ultracentrifuge. The partition cell technique employed was a modification of that of Waugh and Yphantis (6), described fully in another paper (3). Sera were always diluted 1–4 for this type of run, and the resultant protein concentration assumed to be equal on all runs. These runs were made at 52,640 RPM, for 32 minutes at full speed. A deceleration schedule which controlled the movable partition was based upon timing. However, in all runs photographic records of boundary positioning at various times assured that the 19S boundary was well below a reference point indicating the final partition position. By this technique 15 to 21S gamma globulins, and all other components of this or higher S rate collect below the movable partition at the end of the timed run, and moieties of low S rate, including the 7S gamma globulins, are nearly equally distributed in the compartments above and below the platform. Moieties of intermediate S rates (9 to 15S) would probably be in both compartments at the end of the run, according to calculated boundary positions, but this was not determined by actual experiment in this study. Control runs utilizing known 7S and 19S gamma globulins have indicated a high degree of reliability in separating these components (3). It should be emphasized that this type of separation in serum does *not* give the S rate of the agglutinin with accuracy; however, the term 19S for the macroglobulin activity, and 7S for the lower S rate material will be adopted here as a convention for ease of discussion. The sucrose gradient method was adapted from one described by Kunkel, *et al.* (7) and described in more detail in another paper (3). Macroglobulin antibodies are usually found in fractions 1 through 3, and antibody activity of 7S type in fractions 5 through 7.

Chromatographic separation of agglutinin activity was accomplished using diethylaminoethyl (DEAE) cellulose according to a modification of the method of Kochwa, *et al.* (8). The

column was loaded with 1.0 ml serum which had been dialyzed against 0.02 M pH 6.3 phosphate buffer. The first fraction, eluted in 10 steps with pH 6.3, 0.02 M phosphate buffer contained only 7S type gamma-2 globulins as determined by immunoelectrophoresis. The second fraction was eluted with 1.0 M NaCl, in 10 steps and contained the 19S gamma-1 macroglobulins, but quite often small amounts of other gamma globulins. Controls on this method have included runs of isolated mixtures of known 7S and 19S gamma globulin antibodies and of serum containing measured amounts of the components. Immunoelectrophoretic examination of the concentrated eluates was made in each run.

Treatment of sera with the reducing agent, 2-mercaptoethanol, was accomplished by adding this reagent in pH 7.4 phosphate-buffered saline to the serum samples to achieve a final concentration of 0.1 M; a similar amount of buffer was added to control samples. The sera were kept 24 hours at 4°C in sealed tubes, and titered or fractionated as described above.

EXPERIMENTAL

Response of Newborn Rabbits to Immunization.—The frequency and intensity of response of the newborn rabbit to various antigenic stimuli is subject of somewhat conflicting data in the literature. For example, Freund (9) reported that newborn rabbits respond to protein antigens with much lower titers of precipitins than do adult animals, but those injected with sheep cell antigen were as responsive as adults. The agglutinin response to bacterial antigens was found to be intermediate in this respect. Others have reported defective immune capacity in the newly hatched chicken (10), and the newborn human infant (11). Bridges *et al.* (12), have observed that the newborn rabbit responds poorly, or not at all, to protein antigens prior to the 4th week of life. If mycobacterial emulsion adjuvants were employed, responsiveness could be elicited a few days earlier. They correlated the lack of response in this early period of life with the absence of plasma cell development. Eitzman and Smith (13) also found age to be a critical factor in the response of young rabbits to protein antigen. Amounts of bovine albumin which induced immune tolerance before the 14th day of life stimulated an immune response after this age. In contrast to these observations, Sterzl and Trnka (14) reported that a satisfactory response could be regularly elicited in the 5-day-old rabbit by injecting a large volume of bacterial antigen intraperitoneally.

From these various studies, it appeared that further investigation of the quantitative as well as the qualitative aspects of the immune response in this species would be required to understand the nature of any defect in responsiveness in this period of life.

Preliminary experiments indicated marked variation in the response of litters of rabbits to bacterial antigens, depending on dosage, route, and frequency of injection, and upon presence or absence of passively acquired antibody in the serum. Accordingly, after excluding litters which had passively acquired agglutinins, groups of animals were injected with two *Salmonella* vaccines by various routes and schedules, and agglutinins of the flagellar and somatic antigens determined serially.

The results with respect to development of flagellar agglutinins are illustrated by experiments given in Table I, and in Figs. 1 through 5.

Under the conditions employed here, the majority of animals injected from birth produced flagellar agglutinins by the end of the second week of life and

TABLE I

Production of Flagellar Agglutinins in the Newborn Rabbit in Response to Immunization

Group	Antigen, route, and amount injected*	No. of litters	Route of injection	No. of animals	Flagellar agglutinins in serum, by age‡			
					7 to 10 days	14 to 16 days	20 to 24 days	27 to 31 days
1	*S. paratyphi*, 10^9 organisms at birth.	3	i.p. i.p.	6 injected 5 control§	3/6 0/5	5/6 0/5	5/6 0/5	— —
2	*S. paratyphi*, 10^9 organisms i.p., or 3.6 × 10^8 organisms i.d. in 4 sites, on days 1, 3, 7, and 24.	3	i.p. i.d. i.p.	9 injected 7 injected 8 control	3/3 2/3 0/3	6/6 5/5 0/5	— — —	9/9 7/7 0/8
3	*S. paratyphi*, 10^8 organisms i.p. on days 1, 3, 5.	7	i.p. i.p.	18 injected 5 control	2/18 0/5	4/18 0/5	11/18 0/5	12/18 0/5
4	*S. 13, 23, 36: z*, 10^9 organisms i.p., or 3.6 × 10^8 organisms i.d. divided in 4 sites on days 2, 4, 6, and 16.	2	i.p. i.d. i.p.	7 injected 5 injected 5 control	5/5 3/3 0/5	7/7 4/4 0/5	7/7 5/5 0/5	— — —

* Litters of rabbits were bled and screened for passively acquired agglutinins to the indicated antigens. Animals with negative titers were injected with the indicated amount, route and type of bacterial antigen and bled serially at approximately weekly intervals for agglutinin titration.

‡ The numerator indicates those animals having agglutinin titers of 1–10 or greater. The denominator represents the number in the group. The proportion responding is cumulative for each successive age level indicated.

§ Control animals represent litter mates injected with 1.0 ml 0.15 M NaCl alone, but otherwise handled identically to immunized animals.

usually in high titer. The shape of the response curves are those of the classical primary immune response to this group of antigens. The *S. 13, 23, 36:z* strain appeared as antigenic as *S. paratyphi*.

In contrast to the high frequency of production of flagellar agglutinins, only 8 out of 40 animals shown in Table I had a detectable agglutinin response to the somatic antigen during the first month of life. In those in which agglutinins appeared, titers ranged from 1–5 to 1–40, and rarely appeared before the 4th
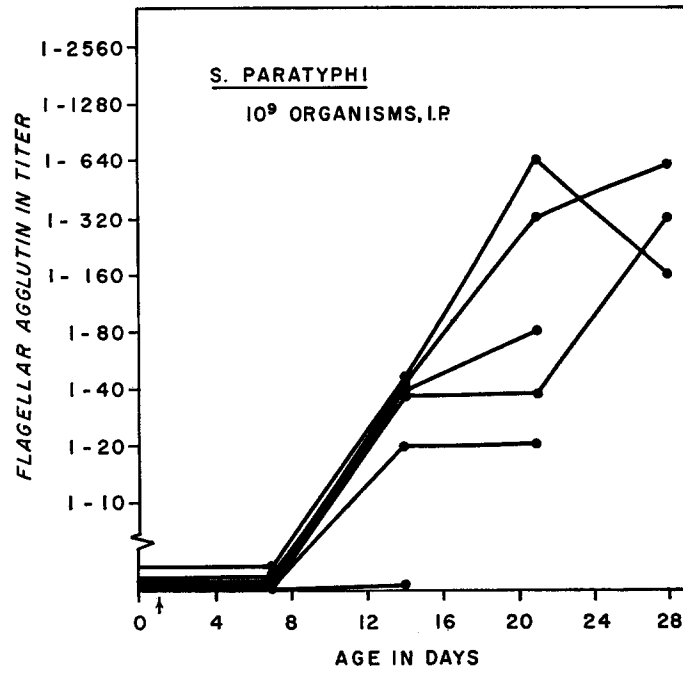
FIG. 1. Flagellar agglutinin response to *S. paratyphi* vaccine in 6 rabbits which received one intraperitoneal injection of 10^9 organisms on the day of birth. 5 of the 6 rabbits produced a significant titer of agglutinins.
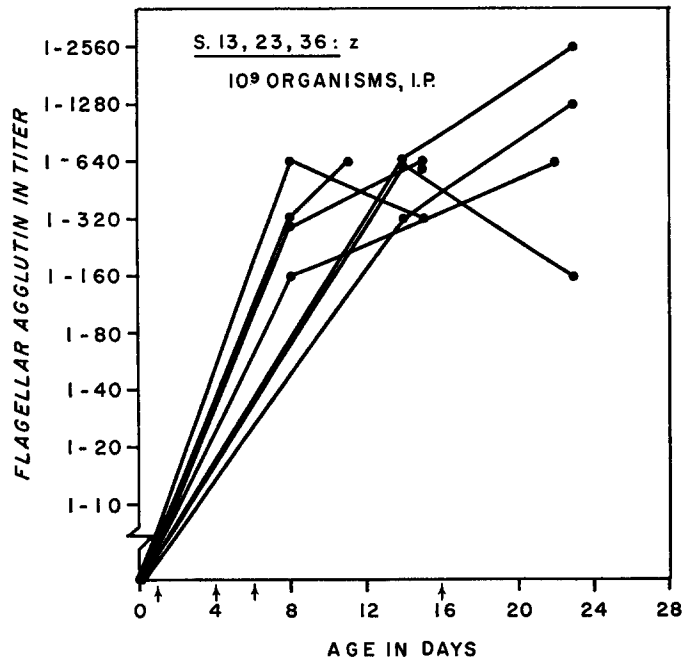

FIG. 2. Flagellar agglutinin response to *S. 13, 23, 36: z* vaccine in 7 rabbits which received 4 intraperitoneal injections of 10^9 organisms on days 2, 4, 6, and 16. All 7 rabbits had produced agglutinins to a titer of 1:160 or greater by day 12.

Fig. 3. Flagellar agglutinin response to *S. paratyphi* vaccine in 9 rabbits which received 4 intraperitoneal injections of 10^9 organisms on days 1, 3, 7, and 24. All 9 rabbits had produced agglutinins to a titer of 1:80 or greater by day 12.
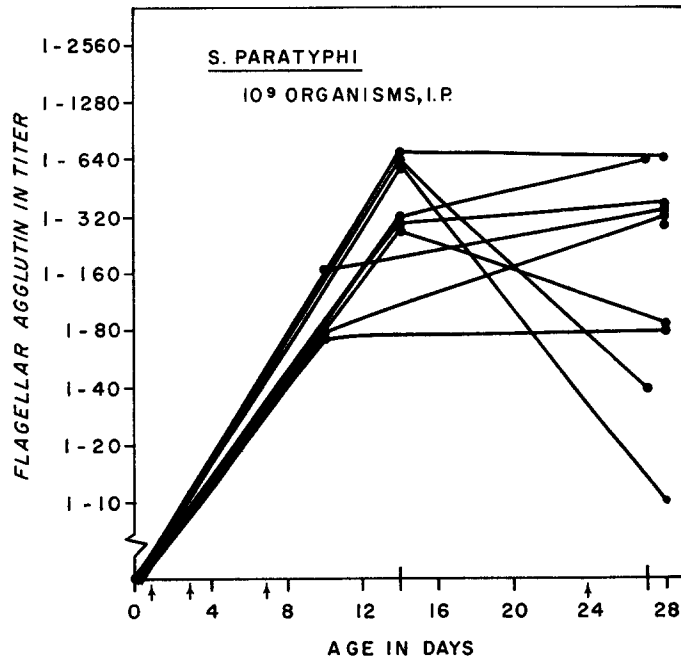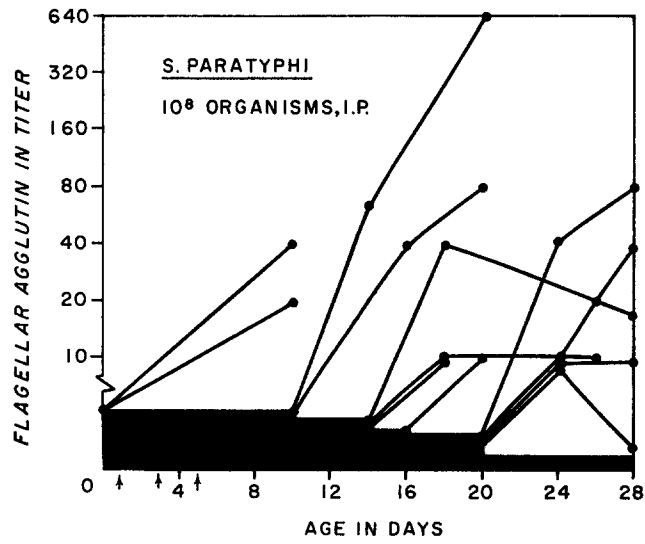

Fig. 4. Flagellar agglutinin response to *S. paratyphi* vaccine in 18 rabbits which received 3 intraperitoneal injections of 10^8 organisms on days 1, 3, and 5. 12 of the 18 animals produced agglutinins.

or 5th week of immunization. The qualitative aspects of these agglutinins are being examined in more detail at the present time.

Both the route and frequency of injection appeared to affect the time of appearance and the amount of antibody produced. Animals in group 1, which received a single intraperitoneal injection at birth, developed on the average lower titers, significantly later than those in groups 2 and 4, which received re-
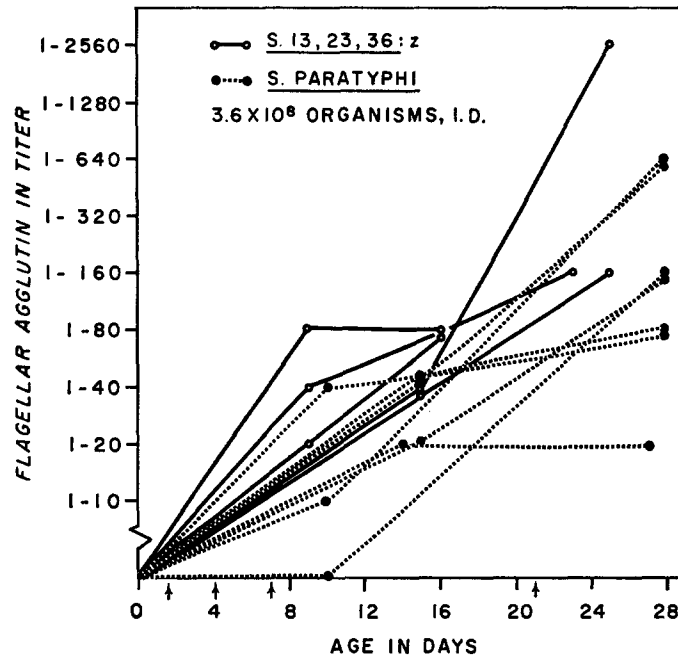

Fig. 5. Flagellar agglutinin response to *S. 13, 23, 36: z* and *S. paratyphi* vaccines given intradermally in 4 sites on days 2, 4, 6, and 21. A total of 3.6 X 10⁸ organisms were given on each day. All of the 12 animals produced a significant agglutinin titer by day 16.

peated injections by this route. The total amount of antigen given in latter groups was three times that of group 1 at the time of the initial response. However, group 3 which received three injections of one-tenth of the number of organisms as did groups 2 and 4, developed lower titers in a lower proportion of animals. In most of those which did respond, the antibody appeared later in the first month than it did in the other groups. The possible significance of this result in discussed later.

Animals in group 2 receiving repeated intradermal injections of approximately one-third the number of organisms also responded with high frequency, but not so early nor with titers comparable to those of their littermates injected by an identical schedule intraperitoneally. The difference in these re-

486 DEVELOPMENT OF IMMUNE RESPONSE

sponses may be related to the route of injection or to the lower dose used. However, this was the maximum practical number of organisms which could be injected into the footpads of the young animals.

In groups 1, 2, and 4 control littermates injected only with 0.15 M NaCl solution, were reared with the injected animals in order to exclude the possibility of development of antibody from natural infection. Control litters were maintained with litters of group 3. These controls were always negative as is shown in Table I.

Under the conditions of this study it appeared that the highest frequency of early high-titer immune response was obtained when the animals were given repeated intraperitoneal injections of antigen from the time of birth, and where the number of organisms was extremely large—on the order of 10^9 in each injection. Even when these conditions were all met, an occasional litter of animals or individual animals unaccountably failed to respond to the identical stimulus which evoked an excellent response in the majority.

Effect of Variation of Antigenic Mass on Flagellar Agglutinin Response in the Newborn Rabbit.—Data from the four types of experiments above suggested that the response to these antigens was related to the dose of organisms given. However, some evidence is available that excessively large doses can inhibit rather than increase the immune response. For example, Kerr and Robertson (15) injecting *Trichomonas foetus* into calves, Buxton (16) and Friedman and Gaby (17) using bacterial antigens in chickens, and Lindorfer and Subrayaman (18) using staphylococcal toxoid in rabbits appeared to show that a large mass of bacterial antigen suppressed immune responsiveness temporarily when given during the neonatal period. More recently, the experiments of Weiss and Main (19) suggest that true immunologic tolerance of diphtheria toxoid may be elicited in the guinea pig. Smith and Bridges (20) on the other hand were unable to detect suppression of immunity with a variety of bacterial antigens given to the newborn rabbit. Although the data recorded in Table I did not appear to lend any support to the possible depressing effect of large amounts of antigens, it was of interest to test the effect of the full practical range of doses of these antigens to ascertain if suppression could be induced.

Accordingly, experiments were designed in which litters of newborn rabbits having no passively acquired antibody, received either 10^8, 10^9, or 10^{10} organisms per ml as a single intraperitoneal injection at birth. The animals were bled at intervals and the agglutinin titers measured. The results of these experiments, illustrated in Table II, are in complete accord with those of Sterzl and Trnka (14). The largest practical dose of antigen which could be given as a single injection gave an enhanced rather than depressed immunologic response. It is still possible that repeated injections of such large amounts would inhibit the immune response, perhaps by specific absorption of antibody activity. This type of experiment was not possible, however, because of the high mortality in newborn animals so injected, presumably from an endotoxin effect.

Characterization of the Flagellar Agglutinin in the Newborn Rabbit.—Previous characterization of flagellar agglutinins produced by newborn human infants indicated that agglutinins having the characteristics of macroglobulin, and possibly a globulin of intermediate size, appeared earliest after starting immunization, and that later in infancy 7S type agglutinins were formed. The mature human revealed a similar sequence of response, although compressed into a much shorter period of time. Similar fractionations have been made on sera from rabbits immunized for these studies, and the sequence of appearance

TABLE II

*Effect of Variation in Dose of Antigen on the Flagellar Agglutinin of the Newborn Rabbit**

Dose of antigen at birth	Animal No.	Reciprocal agglutinin titer, by age				
		0	5	10	15	20
S. paratyphi, 10^{10} organisms i.p.	19-65	0	0	320	640	2560
	19-40	0	0	320	640	2560
	18-99	0	0	320	340	640
	17-94	0	0	80	80	160
S. paratyphi, 10^9 organisms i.p.	19-45	0	0	160	320	320
	17-89	0	0	0	320	1280
	15-55	0	0	0	320	1280
S. paratyphi, 10^8 organisms i.p.	18-98	0	0	0	0	0
	19-60	0	0	0	0	20
	19-47	0	0	0	0	0

* Litters of rabbits were divided into three groups and given either 10^{10}, 10^9, or 10^8 organisms on the 1st day of life, i.p., then bled serially as indicated and the flagellar agglutinin titer determined.

of flagellar agglutinins of varying mobility and molecular weight has been compared in the newborn and adult animal.

Complete characterizations of the activity contained in each newborn serum from these experiments was not practical because of severe limitations on the amount of serum available particularly in early bleedings. However, mobility as indicated by starch block electrophoresis, sedimentation characteristics as determined in the moving partition cell or by sucrose gradient centrifugation, and cellulose column chromatographic fractionation using DEAE cellulose, were performed on multiple samples of agglutinin-containing newborn serum from animals on various injection schedules and at various times after birth. Whenever possible, two methods were used on the same serum. These data gave a consistent composite result.

The results of typical studies are illustrated in Tables III and IV and in

TABLE III

Fractionation of Agglutinin Activity in the Serum* of Individual Rabbits in a Moving Partition Cell at Various Intervals after Injection of Antigen

Age group and injection schedule	Animal No.	Interval after injection	Serum titer	Agglutinin titer in cell compartments after ultracentrifugation‡	
				Upper compartment	Lower compartment
		days			
A. Newborn animals; 10^9 organisms on days 1, 3, and 5.	18-38	0	0	0	0
		16	80	0	64
	18-40	0	0	—	—
		10	40	0	128
		12	40	0	128
	21-23	0	0	—	—
		20	40	0	40
		28	80	0	64
	17-61	0	0	—	—
		18	40	0	32
		26	40	0	64
		28	80	16	64
	17-74	0	0	—	—
		13	80	0	40
		20	640	128	1024
B. Newborn animals; one injection of 10^9 organisms at birth and again at 45 days.	15-48	0	0	—	—
		9	10	0	5
		14	10	0	5
		31	160	0	160
		56	160	20	160
	15-50	0	0	—	—
		9	20	0	5
		14	20	0	20
		31	160	20	20
		56	320	40	320
C. Adult animals given single intradermal injection of 4.5×10^8 organisms.	18-69	0	0	—	—
		3	10	0	8
		6	160	128	512
		8	640	32	512
	15-65	0	0	—	—
		3	0	0	0
		6	80	0	64
		8	640	8	512

* Sera from individual immunized newborn or adult animals were examined serially, as described. Those shown were selected to illustrate the range of patterns of response observed.

‡ Upper or lower compartment indicates the titer of the contents of the centripetal or centrifugal portion, respectively, of the moving boundary cell at end of a standardized 32 minute run at 52,640 RPM. In this interval, the 19S boundary has completely passed the final platform position. The upper compartment accordingly contains no 19S, but only 7S agglutinins in concentration nearly equal to that present in the lower compartment.

TABLE IV

Results of Fractionation of Flagellar Agglutinins Produced by Immunization of Newborn and Adult Rabbits

Procedure*	Interval after immunization	Serum titer	Partition cell titers Compartments		Sucrose density gradient titer‡ Tube No.									DEAE fraction titer	
			Upper	Lower	1	2	3	4	5	6	7	8	9	0.02 M eluate	1.0 M eluate
	days														
A. Newborn animals, received single injection of 10⁸ or 10⁹ organisms, i.p. on 1st day of life.	11	20	0	64	0	+	+	+	0	0	0	0	0		
	18	10	0	16	0	+	+	0	0	0	0	0	0		
	24	320	32	256	+	+	+	+	+	+	+	0	0	10	20
	24	320	0	64	0	+	+	0	0	0	0	0	0		
	25	320	64	256	0	+	+	+	0	0	+	0	0		
	25	160	16	128	0	+	+	0	0	0	+	0	0	10	10
B. Newborn animals, received 3 injections i.p. on days 1, 3, 5.	14	80	—	—				—						0	10
	18	320	32	256	+	+	+	0	+	0	0	0	0		
	18	640	128	1024				—						—	—
	24	640	—	—				—						32	32
	25	320	32	128	0	+	+	+	+	+	+	0	0		
	25	640	256	1024	+	+	+	+	+	+	+	0	0		
	31	160	—	—				—						80	10
C. Adult animal given two injections of 0.4 × 10⁹ organisms, i.d. on days 1 and 3.	0	0	0	0				—						0	0
	5	640	160	640				—						5	80
	7	640	40	640				—						10	80
	10	640	160	640				—						80	40
	16	640	20	160	+	+	+	+	+	0	0	+	+	—	—

* Litters of rabbits were given the indicated injections of antigen and bled at intervals. The results of fractionating the serum of those animals from which sufficient serum was obtained for fractionation by more than one method are shown.

‡ Sucrose gradient fractions contain macroglobulins in tubes 1 to 3 and lower molecular weight materials in fractions 5 to 7.

Figs. 6 *a*, *b*. These data indicate that the earliest agglutinin activity to appear in the newborn rabbit was of a macroglobulin type. Activity was in the lower compartment of the Waugh-Yphantis cell, (Table III), in the lowest tubes of sucrose gradient, or in 1.0 M eluate of the DEAE fractions (Table IV). This early formed agglutinin migrated in the gamma-1 region on electrophoresis and usually in a narrow range of mobility on starch block electrophoresis (Fig. 6 *a*). In some animals, the 7S type of agglutinin activity began to appear between 20 and 30 days of age, but rarely earlier than this; the macroglobulin was
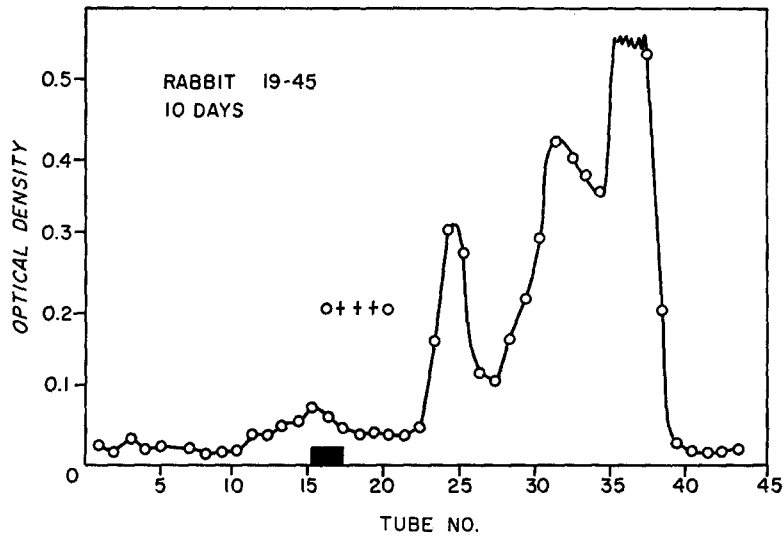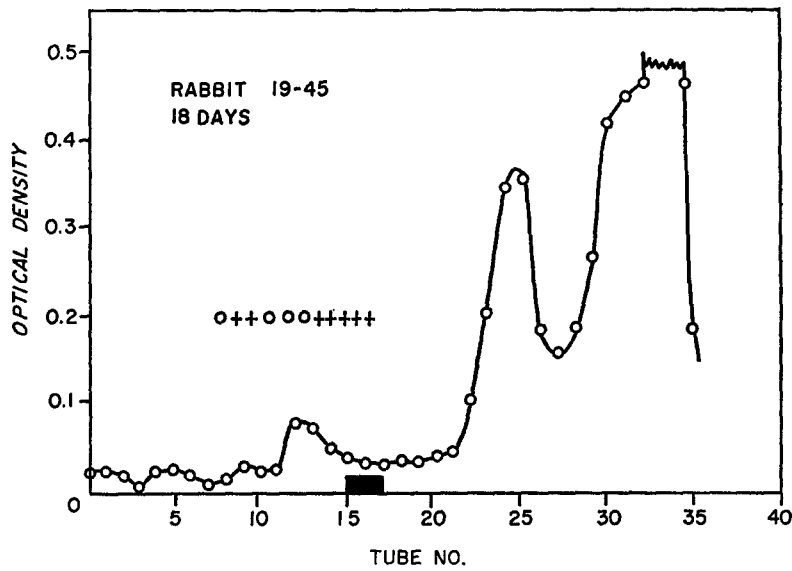
FIG. 6 a

FIG. 6 b

FIG. 6. Starch block electrophoresis of serum specimens, taken from rabbit injected at birth with 1.0 ml 10⁹ S. paratyphi B., (a) shows activity at 10 days of life as a narrow band in the gamma-1 range. (b) shows same animal 8 days later with earliest activity in the gamma-2 range.

still the sole variety at 30 days in some animals. After this age, agglutinin activity was nearly always found in both compartments of the partition cell or throughout the sucrose gradient in both the 1.0 M and the 0.02 M chromatographic fractions, and throughout the gamma-1 and the gamma-2 regions on starch block electrophoresis.

Thus, it appears that the earliest antibody formed to this antigen is macroglobulin-like and that 7S antibody does not make an appearance until the animal is more mature. This sequence is quite similar to that observed in the human infant (1, 3).

TABLE V

*Effect of 0.1 M 2-Mercaptoethanol on the Agglutinin Activity of Sera and Waugh-Yphantis Cell Fractions in Newborn Rabbits**

Animal No.	Interval after birth	Agglutinin titer					
		Untreated			Treated with 0.1 M 2-ME		
		Unfrac-tionated	Upper compart-ment	Lower compart-ment	Unfrac-tionated	Upper compart-ment	Lower compart-ment
	days						
18-40	12	40	0	128	0	0	0
18-38	16	80	0	64	0	0	0
10-48	28	640	16	64	—	16	32
10-62	25	160	16	64	—	16	16

* To aliquots of serum diluted 1-4 or 1-8, in pH 7.2 phosphate-buffered saline were added either 2-mercaptoethanol to final concentration of 0.1 M, or phosphate-buffered saline. After 24 hours at 4° C, the sera were run in the Waugh-Yphantis cell; the fractions titered.

Characterization of the Agglutinin Response of the Adult Rabbit.—Previously unimmunized adult rabbits with no pre-existing agglutinin activity in their sera were injected intradermally in the foot pads with *S. paratyphi* antigen, and bled at frequent intervals. As will be noted in group C of Table III, and group B of Table IV, the agglutinin response to this antigenic stimulus was very rapid. Providing that sampling was sufficiently frequent, the adult rabbit formed only macroglobulin activity between the 3rd and 6th day after the first injection. However, as early as the 5th day, many of the animals studied were also producing the 7S type antibody.

These data are consistent with the several reports in the literature (21-23) which indicate that the early antibody activity produced in the rabbit is one of the higher molecular weight fast gamma globulin type, and that the lower

molecular weight slow gamma globulins appear shortly thereafter. The rapidity of appearance of the lower molecular weight component is the chief characteristic which distinguishes this sequence from that observed in the newborn animal.

Susceptibility of the Newborn Macroglobulin Agglutinin to Reduction by 2-Mercaptoethanol.—Deutsch and Morton (24) have found that macroglobulins, such as isohemagglutinins, lose activity when treated with various reducing agents.

Accordingly, the macroglobulin activity produced by newborn rabbits was measured before and after treatment for 24 hours with 0.1 M 2-ME. Moving partition cell fractions were examined in these and in parallel experiments in which only buffer was added to the serum. Table V gives the result of four typical experiments. The macroglobulin agglutinin activity produced in the 12- or 16-day-old animals was completely lost by the action of this reducing agent. On the other hand, the antibody titer in the 25- and 28-day-old animals, which contained both 7S and 19S components, was not significantly lowered except in the lower compartment containing the macroglobulin. This concentration of 2-mercaptoethanol did not appear to affect the 7S type of antibody activity.

DISCUSSION

The data presented appear to establish that the rabbit is capable of producing a specific agglutinin soon after birth in response to the antigenic stimulus provided by salmonella flagellar antigens. Conditions which were found to favor this process were the intraperitoneal route, repeated injections in the 1st days of life, and a large antigenic mass. In this respect, these findings confirm and extend those of Sterzl and Trnka (14) who found that agglutinin responses were best elicited in 5-day-old animals with very large amounts of antigen.

The extraordinarily large antigenic mass required to elicit antibody formation in the newborn period does not have an adequate explanation at present. It is possible at least, that this massive stimulus provides more than simply a source of antigen—that it acts somewhat as an adjuvant. It has been shown by Condie *et al.* (25), and others (26), that endotoxin does enhance the immune response in adult rabbits to a marked degree. This possibility could be tested by giving mixtures of two organisms each of which was below the apparent 10^8 threshold for numbers required to elicit a response, but each providing the other with any hypothetical adjuvant enhancement.

Another explanation of the requirement for a large antigenic mass may be connected with the comparatively poor inflammatory response mobilized by any stimulus in the neonatal mammal (reviewed in reference 27). Since the inflammatory response represents the first intracellular encounter of the normal organism with an antigenic material, it is possible that the intensive degree of inflammatory stimulus provided by organisms in these experiments was required to initiate the earliest phases of an immune response.

Under the conditions of our experiments a flagellar agglutinin regularly ap-

peared within the first 7 to 10 days of life when the first injections were given at, or shortly after birth. Peak titers achieved between the 14th and 25th day were as high as those observed in adult animals in many instances. Rarely, and then only late in the 1st month of life, antibody capable of agglutinating the somatic antigen of the organisms employed appeared for the first time. It is possible that the relative sensitivity of the flagellar and somatic agglutination techniques may make this an apparent difference only. In a few experiments, using more sensitive hemagglutination methods, however, no agglutinins of the somatic antigen were found: therefore, the differential response to the somatic and the flagellar agglutinin remain unexplained.

Characterization of the flagellar agglutinin revealed two distinct varieties of activity; both appear to be gamma globulins. The earliest to appear, and the only variety in most animals during the first 20 days of life, was a gamma-1 macroglobulin. In its first appearance, the antibody had a narrow spectrum of electrophoretic mobility, but later, this band broadened toward the cathodic end. This is consistent with other studies in this laboratory (28) which have shown that the earliest gamma globulin synthesized by the newborn rabbit exhibits a very narrow range of electrophoretic mobilities as contrasted to the wider band produced later in life.

This macroglobulin has not been isolated, and thus its exact sedimentation characteristics, electrophoretic mobility and other properties can not be established with finality. However, all data presented here are consistent with the interpretation that it is a gamma-1 globulin of the 15 to 20S class, which depends for specific agglutinin activity upon the integrity of relatively labile disulphide bonds. It appears then to be very similar to other macroglobulin antibodies which have been investigated. Unaccounted for at present are the few instances in which activities of intermediate levels in the sucrose gradient appeared during immunization. Some evidence for the appearance of such an intermediate antibody during the immune response of humans has been reported recently by Rockey and Kunkel (29).

During the 4th and 5th weeks of life an additional species of activity appears. This type activity is of gamma-2 mobility, and lower sedimentation rate than the macroglobulin. Its behavior in the partition cell, on cellulose column chromatography, and in sucrose gradients, is entirely consistent with an antibody of the 6 to 7S class. This activity was not susceptible to 2-mercaptoethanol treatment. Until it is isolated and characterized in a more pure state, however, this interpretation must remain somewhat tentative.

Comparable immunization of adult rabbits usually initiated an immune response in the same sequence as that observed in the newborn animal. However, the rapid appearances of the 7S type gamma globulin by the 5th to 7th day stands in marked contrast to the prolonged period—up to 30 days—during which the newborn produces only the macroglobulin.

Two major differences then appear to separate the adult from the newborn

rabbit in their response to this type of antigenic stimulus. First, the macro-globulin type antibody appears a few days earlier in the adult than in the new-born. However, this small difference in timing could be more apparent than real, since comparable bleedings were not obtained because of the high attrition rate associated with multiple bleedings in the very young animal.

More significantly, the two groups differed in the delay between the appearance of the macroglobulin and the smaller gamma globulin type agglutinins. The newborn animal was sufficiently developed at birth to initiate macroglobulin synthesis in respectable titer. The most probable explanation of this lag rests therefore in the delay in its capacity to produce 7S gamma globulin antibody.

The reasons for the delay in 7S gamma globulin synthesis are not clear at present. 7S gamma globulin is transferred quantitatively across the placenta of the rabbit and 19S gamma globulin does not regularly pass in detectable quantity. It is possible that the large excess of 7S gamma globulin acts to repress *de novo* synthesis of this general variety of antibody, until catabolic processes lower the level sufficiently to permit initiation of synthesis. In support of this, it has been shown that individual antibodies passively transferred across the placenta in high titer specifically may inhibit active synthesis of the same type antibody in newborn human infant (3, 30). No data available indicate that a whole molecular species of gamma globulin is inhibited in this way.

The timing of the initiation of 7S antibody synthesis coincides temporally with a constellation of immunologically related events in the 15- to 25-day-old rabbit (31). For example, the rabbit becomes capable of forming antibody to heterologous serum protein antigens during this period regardless of the intensity of the stimulus. At about the same time, susceptibility to induction of immunologic tolerance of heterologous proteins (13) decreases rather abruptly. Protein absorption from the intestine diminishes; and the organ and cell distribution of radioactive antigens—both bacterial and protein—changes. Also correlated in time with these several alterations in the young animal's immune response and its means of dealing with foreign antigenic material, is the first appearance of plasma cells in the lymphoid tissue of the intestine, spleen and lymph nodes (12). During this period of life, rapid metabolic changes and an over-all deficiency of immunologically competent cells are also factors which may be related to the rabbit's capacity to produce 7S-γ-2 antibodies. Though these events are well correlated in time, no evidence yet available links them by any known fundamental mechanism. Understanding the ontogenesis of the immune mechanism, as well as the induction of the tolerant state would be enhanced materially by uncovering a common mechanism underlying all of these immunological phenomena.

SUMMARY

By intensive stimulation with large amounts of *Salmonella* flagellar antigen, newborn rabbits were induced to form high titer flagellar agglutinins usually

by the 7th to 10th day of life. Characterization of the agglutinins at various times during the first 30 days of life revealed that the earliest antibody which appeared was a gamma-1 macroglobulin, and that 7S gamma-2 globulins did not appear until the 4th or 5th week of life. In contrast, the adult animals produced macroglobulin antibodies for only 3 to 5 days before the lower molecular weight variety appeared. The infant macroglobulin appears to be similar in all respects to adult macroglobulin antibodies.

These data are interpreted to indicate that the newborn and adult rabbit differ in their response to this type of stimulus not in timing of macroglobulin antibody production, but chiefly in the prolonged interval, which precedes the development of the capacity for the 7S type response in the newborn animal.

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