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Humoral Bactericidal Systems: Antibacterial Potential of Serum from Young Animals

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The antibacterial potential of fresh serum obtained from young animals during a pre-antibody period of development was assessed against two smooth and two rough strains of gram-negative bacteria. The bactericidal capacity of serum from 3- to 4-week-old guinea pigs and 4- to 5-week-old rabbits was compared with that of serum from adults. Serum from young animals was deficient in natural antibodies, and in conventional dilution assays the bactericidal action was unimpressive, especially against the smooth strains. However, when decimal increments of bacteria were incubated in fresh undiluted serum, killing of both smooth and rough strains proved substantial. This finding may have particular meaning in the very young animal when natural antibodies are at ebb: cellular defense mechanisms may function less efficiently at this time and effect a greater reliance on humoral antibacterial systems.

Serum obtained from several mammalian species during early postnatal development has been reported to lack significant bactericidal activity against smooth strains of enteric bacilli (2, 18). In other studies, serum from 3- to 4-week-old rabbits, guinea pigs, and rats was reported to be essentially nonbactericidal for certain gram-negative bacteria (9, 10). The absence of antibacterial activity was considered a consequence of insufficiencies in natural antibodies that occur during the early postnatal period, i.e., when maternal antibodies wane and before immunological mechanisms are fully developed. In these investigations (9, 10), bactericidal antibody levels were determined by incubation of bacteria in serial dilutions of test sera with exogenous (foreign) complement. While this experimental model provides a highly sensitive means to measure humoral antibodies (8, 18), it may not yield information relevant to an understanding of the contribution of serum bactericidal systems to host defense.

In the present study, bactericidal experiments were designed to approach more closely conditions of a host-parasite encounter. To this end, undiluted or slightly diluted serum was used in bactericidal tests. These tests were carried out within 4 h of blood collection, and no exogenous complement was supplied. Serum from young, immunologically naive rabbits and guinea pigs was used in most experiments to enable an evaluation of bactericidal potential at a time when natural antibody levels are minimal. The results indicate that the bactericidal action of serum from these animals is surprisingly strong against smooth as well as rough strains of enteric bacteria.

MATERIALS AND METHODS

Bacterial cultures. Smooth strains of *Salmonella typhi* Ty2 and *Escherichia coli* O14 and rough strains of *S. typhi* R2 and *Shigella sonnei* phase II were obtained from the culture collection of Institut Pasteur, Paris. Stability of smooth strains was confirmed periodically: distinctions were based on colonial appearance, inagglutinability of cells after boiling in saline for 1 h, and sensitivity and rate of killing by normal human serum. All cultures were transferred weekly on Trypticase soy agar slants.

Normal serum. Preliminary experiments were carried out to determine, in common-stock guinea pigs and white rabbits, the approximate age at which serum levels of certain natural hemagglutinins are minimal. For this purpose, nine littermate rabbits and five littermate guinea pigs were bled at weekly intervals from birth to 3 months of age. At each bleeding, 2.5 ml of blood was taken by heart puncture. Sera were separated from clot by centrifugation, heated at 56°C for 30 min, and stored at −15°C. Each serum sample was tested for heterophile hemagglutinins against normal horse and sheep erythrocytes. In addition, passive hemagglutination tests were done with homologous erythrocytes sensitized (14) with Boivin-type (3) endotoxins prepared from *S. typhi* Ty2 and *E. coli* O14.

Unless otherwise stated, the normal serum used for bacterial killing was obtained by heart puncture from 3- to 4-week-old guinea pigs and 4- to 5-week-old rabbits. Serum was separated from clot by centrifugation and assayed for hemolytic complement (7) and bactericidal activity within 4 h of blood collection. The remaining sera were stored at −15°C in closed tubes; the pH of stored sera was adjusted to 7.3 with dilute HCl before being retested.

Immune sera. Normal serum was collected from adult rabbits before immunization with heat-killed (65°C, 1 h) *S. typhi* Ty2 or *E. coli* O14. For each bacterial strain, four rabbits received four subcutaneous injections of approximately 10^{10} washed cells at weekly intervals. The two antisera were processed from blood collections taken 7 days after the last injection. Adult normal and immune sera were assayed for hemolytic complement and bactericidal activity within 4 h of the time of bleeding.

Serum absorptions. In preparation for bacterial absorption of fresh serum, 5-h broth cultures of bacteria were killed by heating for 1 h at 65°C, and the cells were washed 3 times with sterile saline. Cell pellets were dispersed in chilled serum and held at 10°C for 20 min (3×10^9 to 5×10^9 cells/ml of serum). After centrifugation in the cold, absorbed sera were drawn off and used immediately.

Bactericidal assay. The bactericidal assay is a modified version of an earlier procedure (21). Duplicate portions of fresh serum, ranging from 0.02 to 1.0 ml, were added to sterile tubes (20 by 150 mm) fitted with aluminum caps. Volumes were adjusted to 0.5 or 1 ml with Veronal-buffered saline, after which 0.2 ml of the inoculum containing ~100 bacteria, or decimal increments thereof, was added to each tube. All reactants were kept in an ice bath during preparation. Tubes were placed in a 37°C water bath and incubated for 30 min (rough strains) or 60 min (smooth strains), unless otherwise indicated. After incubation, 7 ml of molten Trypticase soy agar (at 46°C) was added to each tube with a broken-tip pipette to facilitate mixing and to stop the bactericidal reaction. Tubes were immediately positioned at a shallow angle, allowed to gel, and then incubated in an inclined position for 16 h at 37°C. Colonies of surviving bacteria were counted over a translucent light source to determine the percentage kill as compared with controls.

Bacterial inocula were prepared in the following manner. On the day before each experiment, bacteria were transferred to fresh agar slants. On the day of the experiment, isolated colonies were picked from agar slants and transferred to fresh broth and incubated for 5 h at 37°C. The 5-h cultures were then diluted in Veronal-buffered saline (20 mM, pH 7.4) so that the desired number of bacteria would be contained in 0.2 ml. When the inoculum was greater than 100 cells, appropriate dilutions of incubated samples were made before addition of molten agar.

RESULTS

Selection of antibody-deficient sera. To evaluate the antibacterial potential of serum obtained from experimental animals at a time when humoral antibodies were at minimal levels, a preliminary investigation was made of natural hemagglutinin titers in serum from postnatal rabbits and guinea pigs. The results proved helpful in gauging the most suitable time to obtain serum deficient in natural antibodies. Hemagglutinins for normal heterologous erythrocytes and for endotoxin-sensitized homologous erythrocytes were present in serum from both species. Serum titers ranged from 1:10 to 1:20 at birth, but fell to 1:5 or less in 3- to 4-week-old guinea pigs and 4- to 6-week-old rabbits. By the 8th to 10th week of life, hemagglutinins increased (or reappeared) in the serum of both rabbits and guinea pigs. The low point in serum content of natural hemagglutinins was similar to that reported for certain bactericidal antibodies during the early postnatal period (9, 10). Consequently, animals between 3 and 5 weeks old were chosen as the source of antibody-deficient serum for most of the experiments. Hemolytic complement titers of serum from animals of this age were similar to adult levels and ranged from 18 to 33 50% hemolytic complement units per ml for rabbits and 190 to 260 50% hemolytic complement units per ml for guinea pigs.

Bactericidal capacity of fresh serum from young animals. Conventional serum dilution assays for bacterial killing indicated that fresh serum from young rabbits and guinea pigs was moderately active against the two rough strains. However, these sera appeared quite ineffective against the smooth strains, *S. typhi* Ty2 and *E. coli* O14, requiring a minimum volume of 0.5 ml to kill consistently only ~100 bacteria (Table 1). Fresh serum from adult animals exhibited a

TABLE 1. *Bactericidal activity of fresh serum from young animals against S. typhi Ty2 and E. coli O14*

Serum source[a]	Serum vol (ml)	Percent kill of ~10^2 bacteria			
		S. typhi Ty2		*E. coli* O14	
		Avg	Range	Avg	Range
Rabbit[b]	0.50	99	98–100	99	96–100
	0.25	23	0–91	8	0–39
	0.13	7	0–65	0	
Guinea pig[c]	0.50	99	99–100		
	0.25	63	51–85	No killing	No killing
	0.10	32	0–60		

[a] All serum samples were assayed individually.
[b] Three litters, 29 animals 4 to 5 weeks old.
[c] Two litters, 9 animals 3 to 4 weeks old.

similar range of activity when assayed in this manner.

A series of experiments was then carried out to determine the maximum capacity of serum from young rabbits to kill both rough and smooth bacterial strains. Serum pools were used in the remaining experiments, since the average percentage kill of individual sera was very close to that obtained when these same sera were combined in equal quantities. Initially, a minimal amount of pooled fresh serum, just sufficient to kill 100 bacteria, was incubated with decimal increments of smooth and rough strains (Table 2). No significant differences were observed in the percentage kill of 10^2 to 10^6 bacteria. Absorption of rabbit serum with *S. typhi* R2 or *S. sonnei* phase II resulted in twofold reductions in bactericidal capacity against the homologous strains, but the percentage kill remained constant in the minimal volume of absorbed serum required to kill ~100 organisms.

The bactericidal potential of undiluted rabbit serum was determined for all four bacterial strains. Results given in Table 3 demonstrate a remarkable capacity of fresh serum from young rabbits to kill smooth as well as rough strains. In addition, the data suggest that a storage-labile component, apparently unrelated to complement or antibody, participated in the killing of three of the test strains. Similar results were observed with fresh and stored serum from young guinea pigs, except that the smooth *E. coli* strain was insensitive to killing. In fact, fresh serum from young or adult guinea pigs, obtained 24 h or 6 days after a single injection of 10 μg of *E. coli* endotoxin, was likewise inactive against this strain.

The bactericidal capacity of undiluted serum from young rabbits was compared with that of serum from adult normal and immunized rabbits. Only half of the immune sera were capable of killing the homologous strain, whereas the serum from all animals was fully active before immunization. The bactericidal action of fresh normal and immune sera from adult rabbits was similar against the smooth strains and approximately one magnitude greater in capacity than serum from young rabbits (Table 4). Serum from adult rabbits and guinea pigs showed only small losses in bactericidal activity after 1 month of storage at −15°C.

TABLE 4. *Bactericidal capacity of fresh undiluted serum from young, adult, and adult-immunized rabbits*

Pooled serum[a]		No. of bacteria killed	
Source	Vol (ml)	*S. typhi* Ty2	*E. coli* O14
Young	1.0	5×10^6	10^7
	0.5	2×10^6	5×10^6
Adult	1.0	5×10^7	6×10^7
	0.5	3×10^7	4×10^7
	0.3	5×10^6	6×10^6
Immune	1.0	5×10^7	6×10^7
	0.5	3×10^7	4×10^7
	0.3	5×10^6	6×10^6

[a] Minimum serum volumes that killed 99.9 to 100% of the indicated inocula during a 3-h incubation at 37°C; lesser amounts of serum produced limited killing of 10^6 bacteria.

TABLE 2. *Bactericidal capacity of fresh serum from young animals*

Pooled serum		Bacterial strain	Percent killed[a] in 1 h at 37°C				
Source	Vol (ml)		10^2	10^3	10^4	10^5	10^6
Guinea pig	0.25	*S. typhi* Ty2	70.1	74.5	73.2	76.0	63.0
Rabbit	0.08	*S. typhi* R2	99.1	99.8	99.4	99.5	99.1
Rabbit[b]	0.16	*S. typhi* R2	100	99.9	100	99.9	99.8

[a] Bacterial inoculum = 1.2 times indicated increments.

[b] A portion of the serum pool absorbed with *S. typhi* R2 immediately before assay; no change in hemolytic complement titer was evident after absorption.

TABLE 3. *Bactericidal capacity of undiluted serum from young rabbits*

Strain	Inoculum ($\times 10^6$)	Serum vol (ml)	Percent killed	
			Fresh	Stored[a]
S. typhi Ty2	0.8–3.4	0.5–1.0	99–100	0
E. coli O14	1.2–9.6	0.5–1.0	99–100	0
S. typhi R2	4.6–46	0.2–0.5	100	0
S. sonnei phase II	5.5–55	0.1–0.5	100	100

[a] Pooled serum from 10 rabbit littermates was tested on the day of bleeding and after 30 days of storage at −15°C; 50% hemolytic complement units per milliliter averaged 23 for fresh serum and 20 for stored serum.

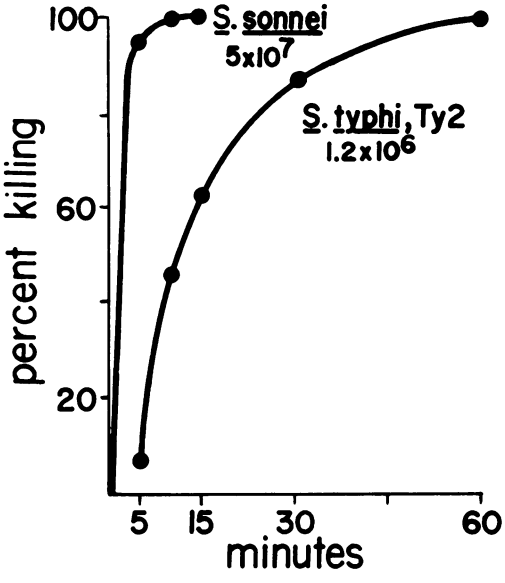

FIG. 1. *Rate of killing in 0.5 ml of undiluted serum from young rabbits.*

The rates of killing of both smooth and rough strains by fresh, undiluted serum from young rabbits are shown in Fig. 1. Fresh serum from adult rabbits produced a somewhat faster rate, reaching >99% kill of the smooth strains in 5 to 10 min less time.

DISCUSSION

The bactericidal activity of serum from several mammalian species has been reported as either lacking or on the decline from birth to about 1 month of age, at which time the serum is incapable of killing certain enteric bacteria (9, 10). The absence or decline in bacterial killing was assessed by a procedure consisting of incubating bacteria in dilutions of test sera in the presence of foreign complement. By contrast, the study of Barta et al. (2) on early postnatal development of serum bactericidal activity in conventional pigs did not show a diminution in antibacterial activity during the first 2 months of life; these authors measured the bactericidal action of undiluted and nominally diluted serum without exogenous complement. Although both animal and bacterial species differences could account for these dissimilar results, it is more likely that basic differences in experimental design were responsible.

It is clear from the present study that the nature of the experimental model adopted for study of the bactericidal potential of serum will markedly affect the outcome and interpretation of results. For example, when bactericidal activity was determined by incremental dilutions of test sera, the data indicated that fresh serum from young rabbits and guinea pigs was ineffective against the smooth strains at dilutions of 1:3 or greater. In fact, consistent killing of only ~100 bacteria required that the serum be diluted no more than 1:2. However, when bacterial inocula were increased by several magnitudes, the full bactericidal potential of fresh, undiluted serum from young animals became evident.

The holding of serum at −15°C resulted in the loss of activity against *S. typhi* Ty2, *E. coli* O14, and *S. typhi* R2 in the absence of critical changes in hemolytic or bactericidal complement; e.g., immune hemolysis and killing of *S. sonnei* phase II remained essentially unchanged. This storage-labile property of serum from young rabbits and guinea pigs, a property also of chick serum (17), suggests that some nonspecific component, in addition to complement, is required for killing of these strains.

The finding that bactericidal action against *S. sonnei* phase II was not reduced during storage at −15°C is more in keeping with results obtained with serum from the adult animals. In these instances, the participation of antibody in the bactericidal action of serum is implicit. Nonetheless, an antibody role in the killing of either of the rough organisms by serum previously absorbed with the homologous strain seems unlikely. In this connection, Sterzl et al. (18) obtained convincing evidence that rough enteric strains can be killed by serum lacking in antibodies. The participation of natural antibodies and other components in the bactericidal action of serum from young animals is considered in the accompanying paper (15), where evidence for both specific and nonspecific systems is presented.

The antibacterial potential of fresh, undiluted serum from adults was somewhat greater than that of serum from young animals and could reflect differences in natural antibody content. On the other hand, immune serum did not differ from normal serum of adult rabbits with regard to the number of bacteria killed, indicating that antibody was not a limiting component in normal serum. The finding that serum from half the adult rabbits became nonbactericidal for the homologous smooth strain after immunization is best explained in terms of the Neisser-Wechsberg effect (11), an effect apparently due to the presence of sufficient specific immunoglobulin G or A to inhibit bactericidal action (1, 4, 12, 20).

The fact that undiluted serum from young guinea pigs failed to kill *E. coli* O14, a strain sensitive to killing by serum from young rabbits, suggested that guinea pig serum lacked antibody

to *E. coli*. However, the inability of post-endotoxin serum from young or adult animals to kill this strain weighs against that possibility. It is not known whether the serum resistance of this strain is associated with K antigen (5) or some other cell surface structure that might interfere with the action of bactericidal components in guinea pig serum but not in rabbit serum.

In spite of a long interest and a voluminous literature on the subject of humoral bactericidal systems (16), the importance of serum or plasma as a major barrier against bacterial invasion is not fully appreciated. Since mammals generally survive the postnatal period without untoward difficulties with infectious agents, humoral bactericidal systems may well assume critical importance during early maturation, particularly if transient deficiencies in humoral antibodies result in reduced effectiveness of cellular defense mechanisms.

This investigation shows that under suitable experimental conditions, fresh serum containing minimal amounts of antibody manifests a considerable capacity to kill both smooth and rough strains of gram-negative bacteria. Evaluations of serum bactericidal potential in terms of the present experimental model and other appropriate models (6, 13, 19) should contribute to a greater appreciation of humoral mechanisms in defense against many enteric bacilli regarded as resistant to killing by normal serum.

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