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Immunity to *Salmonella gallinarum* During Ontogeny of the Chicken

III. BACTERICIDAL ANTIBODY

J. B. SOLOMON*

Department of Microbiology, University of Adelaide, South Australia

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Summary. The bactericidal activity of chicken serum before and after injection of 1–35-day-old chickens with *Salmonella gallinarum* has been studied. The bactericidal action of immune serum is temperature-dependent. Treatment of both normal and immune serum with mercaptoethanol, by heating to 56°, produced a ten-fold reduction in bactericidal activity. The ingested yolk contained bactericidal factors at 1 day of age. Bactericidal activity of normal serum declined very slowly during the first 5 weeks of development. Bactericidal activity released by injection of *S. gallinarum*, showed some specificity for the somatic-O antigens and a high degree of species specificity. Injection of *S. gallinarum* into 1-day-old chicks resulted in the rapid release of a high level of bactericidal activity into the circulation. The bactericidal antibody response to an injection of *S. gallinarum* increased from 4 to 35 days of development. In chickens of 3 weeks of age, or older, induced bactericidal antibody remained at a high level for several weeks.

INTRODUCTION

Neither opsonins nor agglutinins contribute in more than a minor way towards resistance to *Salmonella gallinarum* infection in young chickens (Solomon, 1968a, b). An obvious requirement for the immunological reaction essential for resistance to bacteria is that it be capable of killing the organisms. It was anticipated that the onset of resistance in developing chickens (Solomon, 1968a) could be equated with an increasing ability to produce bactericidal antibody. However, when 1-day-old chicks were injected with bacteria they rapidly released bactericidal factors into the circulation; yet chicks at this age are immunologically incompetent to *S. gallinarum* and are thus susceptible to infection. It is suggested that bacteria initiate a two-stage process. Firstly, the rapid release of bactericidal factors, which may be somewhat non-specific, and secondly, the induction of bactericidal antibody formation by lymphoid cells.

MATERIALS AND METHODS

Injection with Salmonella gallinarum

The methods of rearing White Leghorn chickens and injecting them with *S. gallinarum* have been described previously (Solomon, 1968a). *S. gallinarum* 9240 and its avirulent mutant, *S. gallinarum* 9S were kindly supplied by Dr H. W. Smith, Lilystone Hall, Stock,

* Present address: Department of Bacteriology, University of Aberdeen, Scotland.

Essex. All other bacteria were kindly given by Dr Nancy Atkinson, Department of Microbiology, University of Adelaide. Log phase cultures of bacteria were counted in a Petroff–Hauser counting chamber prior to injection and the number of viable bacteria confirmed by plating on agar and colony counts.

Tests for bactericidal activity

Blood was obtained by cardiac puncture, for the first 5 days after injection, or in other experiments, at weekly intervals. After clotting, the serum was obtained by centrifugation and stored at $-20°$. All sera were assayed within 1 week, since bactericidal activity slowly decayed at $-20°$. The bactericidal assay consisted of incubating small numbers of bacteria with either normal or immune serum and a source of complement. The serum to be tested (0·1 ml) and fresh serum (0·1 ml) from an adult Australorp chicken were added to Difco broth (0·7 ml) in sterile stoppered bottles. Approximately 400 bacteria in 0·9 per cent saline (0·1 ml) were added. Control bottles contained normal saline (0·1 ml) in place of the serum. The bottles were incubated for 2 hours at 37°. An aliquot (0·1 ml) from each bottle was plated on agar, incubated overnight and the number of colonies counted. Titrations were usually made with undiluted serum and 10^{-1} and 10^{-2} dilutions in normal saline. Titres of bactericidal antibody were calculated by plotting the log serum concentration against probits of the percentage of bacteria surviving. End points were taken at 50 per cent bacterial survival (Landy, Michael and Whitby, 1962).

Complement

Complement is essential for bactericidal action of the serum under test. Adult chicken serum is highly suitable as a source of complement, as it contains only traces of natural bactericidal antibody (Fig. 1). This obviated difficulties associated with absorption of all bactericidal antibody from serum used as a source of complement (Šterzl, Kostka and Lanc, 1962) and enabled the assay system to approximate to the *in vivo* situation. The addition of guinea-pig serum (after absorption with *S. gallinarum* 9S and ultrafiltration) together with adult chicken serum, did not affect the bactericidal titre of immune serum.

RESULTS

The action of bactericidal factors in immune serum is temperature-dependent (Table 1).

TABLE 1

TEMPERATURE-DEPENDENCE OF BACTERICIDAL ACTIVITY OF IMMUNE SERUM

Dilution of immune sera	No. of *Salmonella gallinarum* 9S colonies after treatment with:			
	Serum A*		Serum B†	
	4°	37°	4°	37°
10^{-2}	141	2	156	14
10^{-3}	165	31	141	355
10^{-4}	177	215	140	602
Normal saline only	144	718	144	718

* Serum from 5-week-old chickens 4 days after injection with 10^8 live *S. gallinarum* 9S.

† Serum taken from the above experiment at 7 days after injection.

TABLE 2

EFFECT OF PRE-TREATMENT OF SERA WITH HEAT OR 2-MERCAPTOETHANOL ON THE BACTERICIDAL ACTIVITY FOR *Salmonella gallinarum* 9S

Treatment	Bactericidal titre of sera	
	Normal*	Immune†
Untreated	$1{\cdot}6\times10^{-2}$	6×10^{-2}
0·1 M 2-Mercaptoethanol (37° for 30 minutes)	$<10^{-1}$	6×10^{-1}
Heat (56° for 30 minutes)	1×10^{-1}	2×10^{-1}

* Normal 1-day-old chick serum.
† Serum from 2-day-old chicks injected 24 hours earlier with *S. gallinarum* 9S.

In the absence of bacterial growth (at 4°) no killing occurred (also Landy *et al.*, 1962). Treatment with 2-mercaptoethanol, or heating to 56°, produced at least a ten-fold reduction in bactericidal activity of both normal and immune sera from young chicks (Table 2). The susceptibility of bactericidal activity of normal serum to heating at 56° is well known (Skarnes and Watson, 1957; Michael, Whitby and Landy, 1962). In mammals, immune bactericidal antibody is stable at 56°; however, immune haemagglutinins of chicken have recently been shown to be thermolabile at 63° (Delhanty and Solomon, 1966). Chicken sera did not contain an inhibitor of bactericidal activity, as no pro-zone effect (Neisser and Wechsberg, 1901) was ever observed.

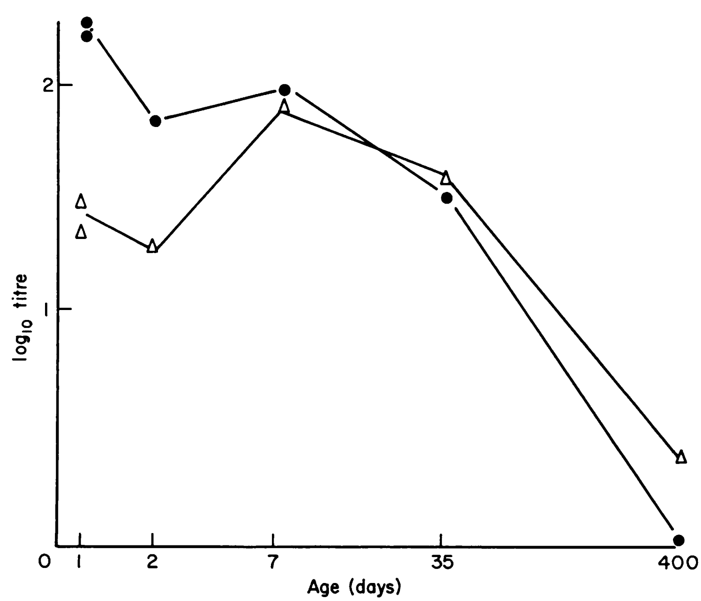

FIG. 1. Bactericidal activity to *Salmonella gallinarum* in normal sera during development. Virulent strain (△); avirulent strain (●).

BACTERICIDAL ACTIVITY OF NORMAL SERA DURING DEVELOPMENT

The yolk sac of 1-day-old chicks contained bactericidal activity. Bactericidal titres of a suspension of yolk in normal saline (1:1) were $1{\cdot}5 \times 10^{-2}$ for *Salmonella gallinarum* 9S and 4×10^{-1} for *S. adelaide*. In normal serum, bactericidal activity for *S. gallinarum* 9S declined slowly throughout development and was still relatively high at 5 weeks of age (Fig. 1). One- and 2-day-old chick sera had less bactericidal activity for *S. gallinarum* 9240 than *S. gallinarum* 9S, but from 1 week of age titres for both strains were similar.

BACTERICIDAL ACTIVITY RELEASED BY INJECTION OF *Salmonella gallinarum*

Bactericidal activity, rapidly released into the circulation within 24 hours after injection of 5-week-old chicken with *S. gallinarum* 9S, showed only slight specificity for the somatic-O antigens (Table 3). A considerable proportion of the induced bactericidal activity reacted

TABLE 3

SPECIFICITY OF BACTERICIDAL ACTIVITY OF SERUM OF 5-WEEK-OLD CHICKENS INJECTED 24 HOURS EARLIER WITH 10^9 LIVE *Salmonella gallinarum* 9S

Bacteria	Somatic-O antigens	Bactericidal titre		Difference between normal and immune
		Immune	Normal	
S. gallinarum 9S	1, 9, 12	2×10^{-3}	3×10^{-1}	$1{\cdot}7 \times 10^{-3}$
S. gallinarum 9240	1, 9, 12	2×10^{-3}	4×10^{-1}	$1{\cdot}6 \times 10^{-3}$
S. strasbourg K 627	9, 46	1×10^{-3}	$<10^{-1}$	$1{\cdot}0 \times 10^{-3}$
S. typhimurium (Edwards 9)	1, 4, 7, 12	1×10^{-2}	$<10^{-1}$	$1{\cdot}0 \times 10^{-2}$
S. adelaide, 1	35	3×10^{-2}	$<10^{-1}$	$3{\cdot}0 \times 10^{-2}$
Vibrio alkaligenes N.C.T.C. 440	—	3×10^{-1}	1×10^{-1}	$2{\cdot}0 \times 10^{-1}$
Bacillus sp.	—	2×10^{-2}	2×10^{-2}	0

with *Salmonella* antigens other than the somatic-O antigens (see *S. adelaide*), possessed by the immunogenic strain. *S. gallinarum* did not induce much, if any, bactericidal activity to two other species of bacteria (*Vibrio alkaligenes* and *Bacillus* sp.).

High levels of bactericidal activity appeared in the serum 24 hours after injection of 1-day-old chicks with only small numbers of live *Salmonella gallinarum* 9240 (Fig. 2). A large dose of killed bacteria was not quite as efficient in stimulating this response (Fig. 2). One-day-old chicks injected with 700 live *S. gallinarum* 9S released more bactericidal activity in the early phase of the response (Fig. 3) than the virulent strain. The overall responses of 5-week-old chicken to both strains were of similar magnitude (Fig. 4). The extent of such responses in 1-, 4-, 7- and 35-day-old chickens are compared in Table 4. The level of bactericidal activity in normal sera at the time of injection is deducted from the geometric means of titres measured during the 5 days following injection. The increasing amount of bactericidal activity released by injection of *S. gallinarum* from 4 to 35 days of age suggests that this may be a form of immunological maturation.

In previous experiments on agglutinin production by chickens injected with *S. gallinarum* (Solomon, 1968b), sera were obtained by bleeding at weekly intervals; a second injection

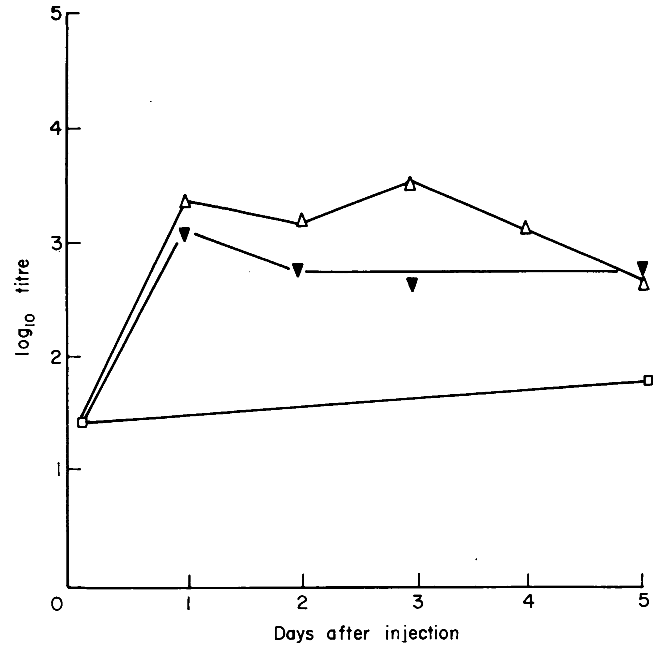

FIG. 2. Bactericidal activity to *Salmonella gallinarum* in serum of 1-day-old chicks after injection with 176 live (△) and 10^8 killed (▼) virulent strain. Normal serum (□).

TABLE 4

RELEASE OF BACTERICIDAL ACTIVITY AFTER INJECTION OF *Salmonella gallinarum* INTO CHICKENS OF 1–35 DAYS OF AGE

Age (days)	Strain	Dose	Bactericidal titre: Mean daily titre during first 5 days after injection	Normal	Difference between normal and immune
1	9240	176	1×10^{-3}	3×10^{-1}	7×10^{-1}
	9S	700	6×10^{-3}	2×10^{-2}	4×10^{-1}
4	9240	900	7×10^{-1}	4×10^{-1}	3×10^{0}
	9S	1×10^{3}	6×10^{-2}	9×10^{-1}	7×10^{0}
7	9240	3×10^{3}	8×10^{-2}	7×10^{-1}	1×10^{-1}
	9S	5×10^{8}	9×10^{-2}	8×10^{-1}	1×10^{-1}
35	9240	1×10^{9}	3×10^{-3}	5×10^{-1}	8×10^{-1}
	9S	1×10^{9}	3×10^{-3}	4×10^{-1}	9×10^{-1}

of bacteria was given 4 weeks after the first and the secondary response followed for 3 weeks. A large number of such sera were assayed for bactericidal activity. Bactericidal titres remained remarkably constant in sera from chickens immunized from at least 3 weeks of age. In a typical experiment (Table 5) titres of the secondary response were no greater than those of the primary response. Landy, Sanderson and Jackson (1965) also found that no priming for bactericidal antibody occurred with relatively large doses of bacterial antigen.

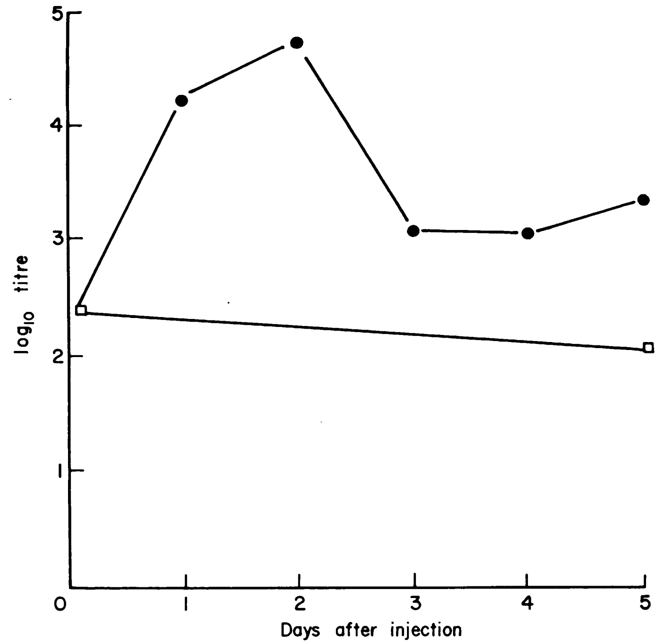

FIG. 3. Bactericidal activity to *Salmonella gallinarum* in serum of 1-day-old chicks after injection with 700 live avirulent strain (●). Normal serum (□).

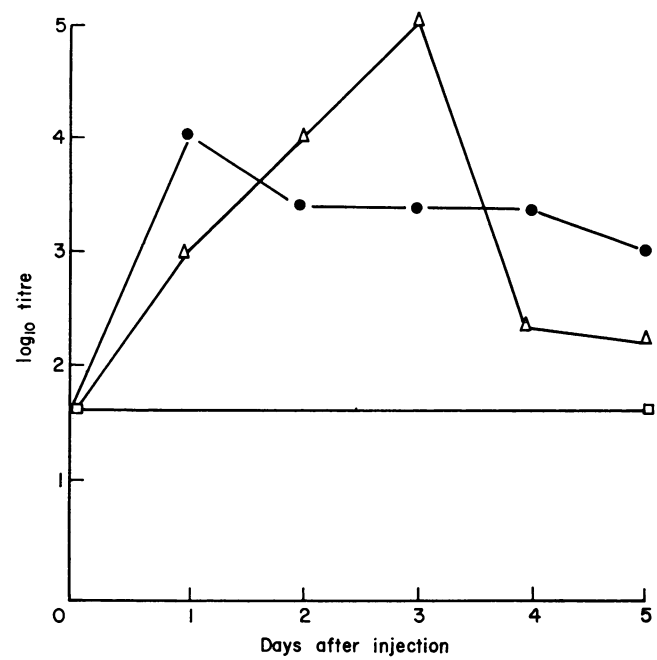

FIG. 4. Bactericidal activity to *Salmonella gallinarum* in serum of 5-week-old chickens after injection with 10° live virulent (△) or avirulent (●) strain. Normal serum (□).

TABLE 5

PRIMARY AND SECONDARY* RESPONSES OF 4-WEEK-OLD CHICKENS TO 10^8 LIVE *Salmonella gallinarum* 9S

Time after injection (weeks)	Bactericidal titre	
	Primary response	Secondary response
1	8×10^{-4}	1×10^{-3}
2	1×10^{-3}	1×10^{-3}
3	5×10^{-3}	1×10^{-4}

* Injected at 8 weeks of age.

DISCUSSION

Sometimes bactericidal antibody has been reported to be specific for its antigen, in other cases, non-specific (Skarnes and Watson, 1957). However, when careful titrations of serum with varying amounts of homologous or heterologous strains of bacteria were made, bactericidal antibody specific for the homologous strain was the first to be absorbed. When the number of bacteria was increased, heterologous antibody was also absorbed (Michael *et al.*, 1962). In the present experiments, a considerable proportion of the immune bactericidal antibody was not specific for the somatic O-9 and O-12 antigens possessed by the immunogen, but showed some species specificity. A high degree of specificity for the O-9 antigen is demonstrable with immune agglutinins (Solomon, 1968b).

Normal sera of newborn mammals do not contain bactericidal activity for *Salmonellae*, but natural bactericidal antibody to this species of organisms appears within 1 week of birth in several species (Šterzl *et al.*, 1962; Michael *et al.*, 1962). However, bactericidal activity for *Salmonellae* is present in the yolk of the ingested yolk sac of 1-day-old chicks and also occurs in their serum. Bactericidal activity to *S. gallinarum* declined very slowly with age and was virtually absent from adult serum. Severens, Roberts and Card (1944) found that bactericidal activity to *S. pullorum* decreased during the 1st week after hatching. On the other hand, Collins (1967) detected little bactericidal action against *S. gallinarum* in normal chicken serum throughout development.

It is well known that endotoxins of Gram-negative bacteria stimulate the release of bactericidal factors into the circulation of adult animals (Fischer, 1959). This bactericidal activity, which appears as early as 3 hours after injection of endotoxin, may be raised to two to three times the normal level for 48 hours and returns to normal after 96 hours (Michael, Whitby and Landy, 1961). The non-specific nature of this type of reaction is demonstrated in the following experiment of Turner, Jenkin and Rowley (1964). Living *Salmonella* injected into mice induced a rapid release of all classes of serum proteins; levels were 70 per cent above normal after 24 hours, but had returned to normal by 5 days. This suggests that endotoxin, whether it is injected as such, or is released from dead bacteria *in vivo*, stimulates a rapid non-specific release of proteins, possibly due to cell damage. Some of this protein is bactericidal in the presence of complement. This transitory release precedes the production of bactericial antibody by lymphoid cells; it is the specific antibody which will be maintained in the circulation for long periods.

One-day-old chicks are considered to be only slightly competent at producing antibody, yet an injection of *S. gallinarum* results in the rapid release of considerable bactericidal activity into the serum. The rapidity of release of such factors might suggest that the contents of the yolk sac were expelled into the circulation. However, it is unlikely that this released bactericidal activity is necessarily of maternal origin as pre-colostral piglets, which have received no maternal antibody, also show a similar release when injected with endotoxin within 7 days of birth (Šterzl *et al.*, 1962). It is possible that 1-day-old chicks release large amounts of non-specific bactericidal factors, which are not derived from the small population of lymphoid cells present at this age. Although traces of immune opsonizing antibody have been detectable at hatching, this was only after previous immunization with heterologous erythrocytes during embryonic life (Solomon, 1966).

Neither the high bactericidal activity of normal serum, nor its supplementation by further release of such activity, affords much protection to infection of 1-day-old chicks with *S. gallinarum*. Conversely, 4-day-old chicks, which are highly resistant to infection with the avirulent strain (Solomon, 1968a) have only a limited ability to elaborate bactericidal antibody. Moreover, while the magnitude of the bactericidal response may increase during 4–35 days of age, it is never greater than that of the highly susceptible 1-day-old chick. Thus, bactericidal antibody cannot be associated with the onset of actively acquired resistance to infection with *S. gallinarum* in young chickens.

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