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Immunity to *Salmonella gallinarum* During Ontogeny of the Chicken

II. INDUCTION OF TOLERANCE OR PRIMING BY SINGLE DOSES OF LIVE OR KILLED BACTERIA

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Summary. Partial tolerance was induced in cells forming agglutinins by live or killed, virulent or avirulent strains of *Salmonella gallinarum* in chick embryos, 1-day and 1-week-old chicks. Tolerance was induced by a single injection of only 100–200 live virulent organisms in 1-day-old and 1-week-old chicks. However, 10^9 avirulent organisms were required to induce a similar degree of tolerance in 1-week-old chicks. The primary agglutinin response paralleled the relative increase in spleen weight during early development and increased only slightly after 5 weeks of age. Priming to live bacteria occurred at 2 weeks of age; the magnitude of the secondary response showed little further increase with age. The induction of partial tolerance in cells producing agglutinins did not increase susceptibility to infection and further emphasizes that agglutinin production is not a protective immune mechanism for *S. gallinarum* infection.

INTRODUCTION

Within 5 days after hatching, chicks become highly resistant to infection with an avirulent strain of *Salmonella gallinarum*. The onset of resistance can be correlated with the onset of immunological competence to react to other particulate antigens which occurs at this time. However, these young chicks are still susceptible to infection with a virulent strain of this organism and do not acquire a high level of resistance until 5 weeks of age. It appears that a quantitatively higher degree of immunity is required to furnish resistance to the virulent strain (Solomon, 1968a).

One-day-old chicks are highly susceptible to infection with both strains of *S. gallinarum*. Susceptibility to infection may not only be an inability to react immunologically, but may also involve the induction of tolerance (Felton, 1949). In this context, it is possible that the virulent strain is a better tolerogen than the avirulent mutant, which might further account for its virulence. Tolerance to killed *S. pullorum* has been successfully induced in 15-day-old chick embryos, but surprisingly, not in older embryos (Buxton, 1954). The induction of tolerance to a single injection of live bacteria has not previously been reported. Very small numbers of *S. gallinarum* have now been shown to induce partial tolerance in cells responsible for the production of agglutinins. Tolerance may be induced in 1-day-old and 1-week-old chicks, but in the case of the avirulent strain much larger doses of bacteria were required to induce tolerance in 1-week-old chicks.

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MATERIALS AND METHODS

Chickens

Chickens were hatched from eggs obtained from a closed flock of White Leghorns. Young chickens were housed in heated cages for the 1st week after hatching and then kept in wire-floored cages at 21° with food and water *ad libitum*. At 8 weeks of age the chickens were transferred to similar cages in the open.

Salmonella *cultures*

S. gallinarum 9S [1,9,12; –] is an avirulent mutant derived from the parent virulent strain of *S. gallinarum* 9240 [1,9,12; –]; both were kindly supplied by Dr H. W. Smith, Lilystone Hall, Stock, Essex. The LD_{50} for these two strains in chicken up to 5 weeks of age has been reported by Solomon (1968a). All other strains of *Salmonella* were kindly supplied 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 and colony counts. Bacteria were killed by standing subcultures in boiling water for 2 hours (Buxton, 1954).

Injection of bacteria

Methods of injection of embryos and young chickens have previously been described (Solomon, 1968a). The dose most frequently used to elicit both primary and secondary responses was 1×10^8 bacteria. In some experiments, higher doses of bacteria were injected, but these produced only slight differences in agglutinin titre. As embryos were killed by less than five live bacteria, they were injected with $2{\cdot}5 \times 10^5$ heat-killed *S. gallinarum* 9S or *S. pullorum* (the dose previously used by Buxton, 1954). Chickens were challenged at 5 weeks of age with 1×10^8 live bacteria. When chickens were older than 1 week at the time of the first injection they were challenged 4 weeks later.

Agglutination tests

Blood was collected by cardiac puncture, stood at room temperature for a few minutes until clotted, then centrifuged for 15 minutes. An equal volume of serum from each bird was pooled and stored at −20°. Doubling dilutions of serum were made in tubes using, initially, 0·1 ml normal saline and 0·1 ml serum. A suspension of approximately 5×10^8 bacteria in normal saline (0·1 ml) was then added. Tubes were incubated for 4 hours at 37° and read after storage overnight at 4°. The agglutination end-point was taken as the last tube to show a definite agglutinated 'mat' of bacteria over the whole bottom of the tube. Agglutinations were performed in duplicate on each batch of pooled serum. Some sera were pre-treated with 2-mercaptoethanol (ME) as follows: equal volumes of serum (0·1 ml) and 0·2 M ME were mixed in a stoppered tube and incubated for 1 hour at 37°. The agglutinin titre of this mixture was measured in the same way as that used for untreated serum.

Quantitation of agglutinin responses

Primary responses, when not shown in full, are expressed as the geometric mean of titres of pooled sera (eight birds in each group) taken at 1, 2, 3 and 4 weeks after the primary injection. Secondary responses are expressed as the geometric mean of titres of pooled sera taken at 1, 2 and 3 weeks after the second injection.

RESULTS

The agglutinations were specific for the somatic O-antigens possessed by the immunogen, *Salmonella gallinarum*. Antiserum to *S. gallinarum* 9S showed no difference in ability to agglutinate *S. gallinarum* 9S or 9240, *S. pullorum* and *S. enteritidis* (Table 1); all these

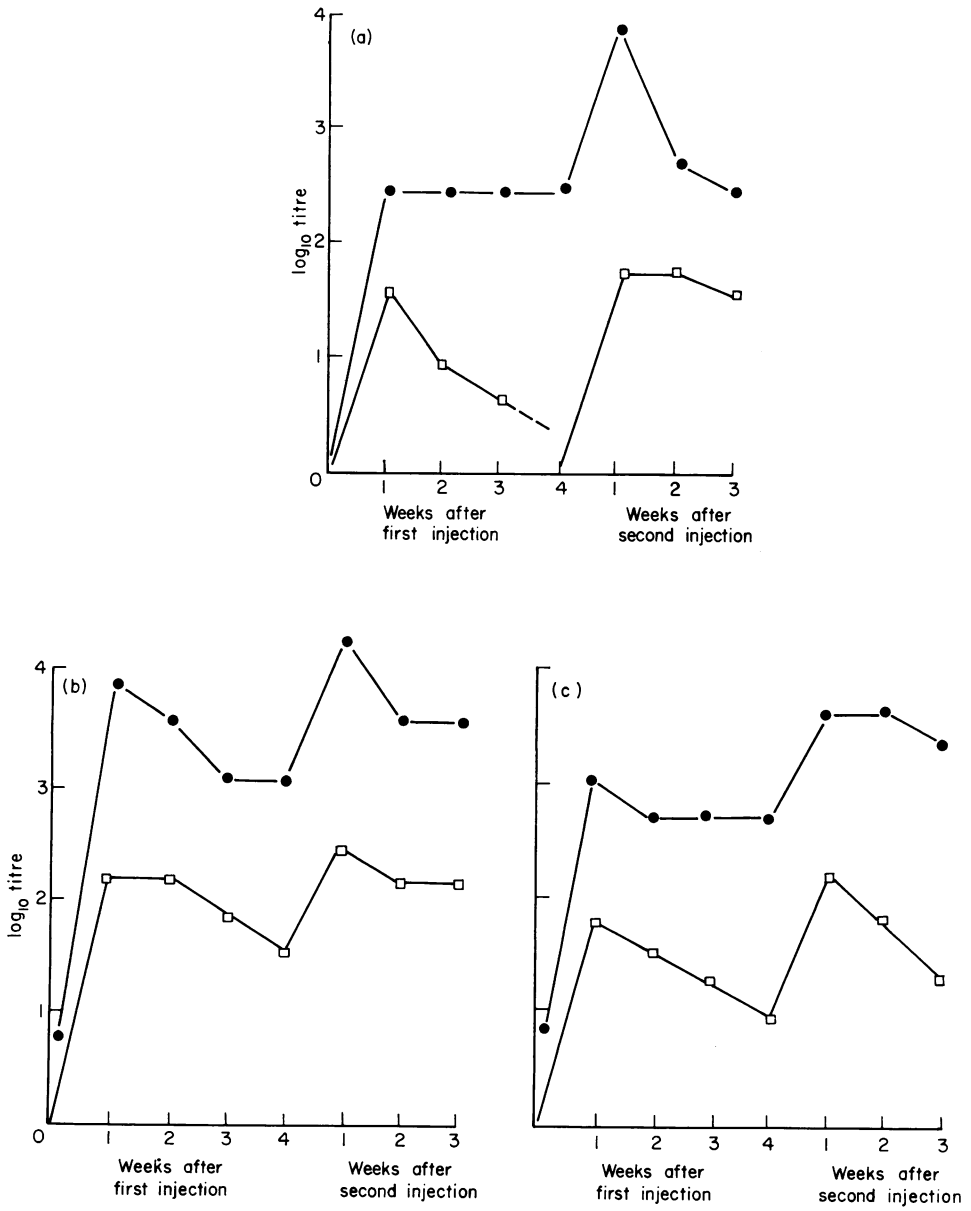

FIG. 1. Mercaptoethanol-resistant antibody in the primary and secondary agglutinin responses to live *Salmonella gallinarum* 9S in: (a) 3-week-old chickens, and (b) 25-week-old chickens. (c) Mercaptoethanol-resistant antibody in the primary and secondary agglutinin response to live *S. gallinarum* 9240 in 25-week-old chickens. Untreated sera (●); mercaptoethanol-treated sera (□).

TABLE 1

SPECIFICITY OF AGGLUTININS FOR THE SOMATIC O-9 ANTIGEN

Salmonella agglutinated	Somatic O-antigens	Reciprocal agglutinin titre using anti-*S. gallinarum* 9S serum
S. gallinarum 9S	1, 9, 12	256
S. gallinarum 9240	1, 9, 12	256
S. pullorum P.40	1, 9, 12	256
S. strasbourg	(9), 46	128
S. typhimurium	1, 4, 5, 12	16
S. adelaide	35	4

bacteria possess identical major groups of somatic O-antigens (1,9,12). When somatic O-9 antigen was absent (*S. typhimurium* and *S. adelaide*) little agglutination occurred.

Mercaptoethanol (ME)-resistant agglutinin was measured in the primary and secondary responses of 3-week-old chickens to 1×10^8 live *S. gallinarum* 9S (Fig. 1a). The highest proportion of ME-resistant agglutinin was about 10 per cent of the titre of untreated serum. However the amount of ME-resistant agglutinin in 25-week-old chickens was only 1 per cent of the titre of untreated serum (Fig. 1b and c). The virulent strain (*S. gallinarum* 9240) elicited significantly lower titres of ME-resistant and ME-susceptible agglutinin in both the primary and secondary responses of 25-week-old chickens (Fig. 1c). The nature

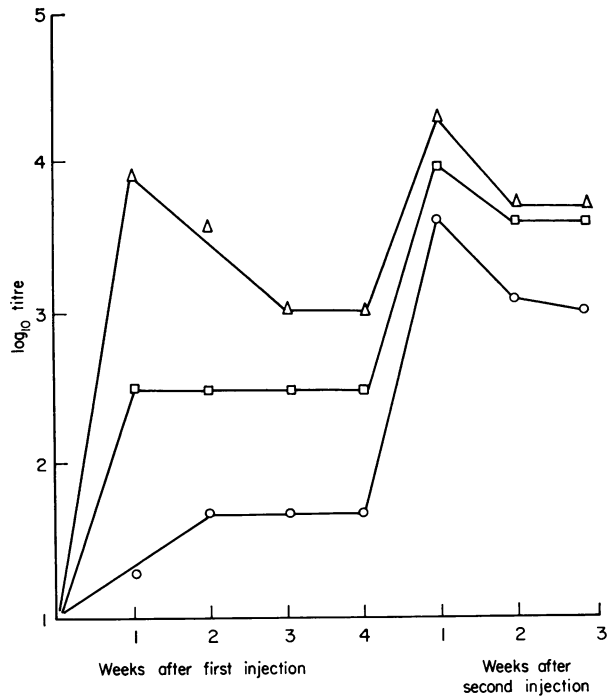

FIG. 2. Primary and secondary agglutinin responses of 2- (○), 3- (□) and 25-week-old (△) chickens to live *Salmonella gallinarum* 9S. Dose of 10^8 bacteria for first and second injection.

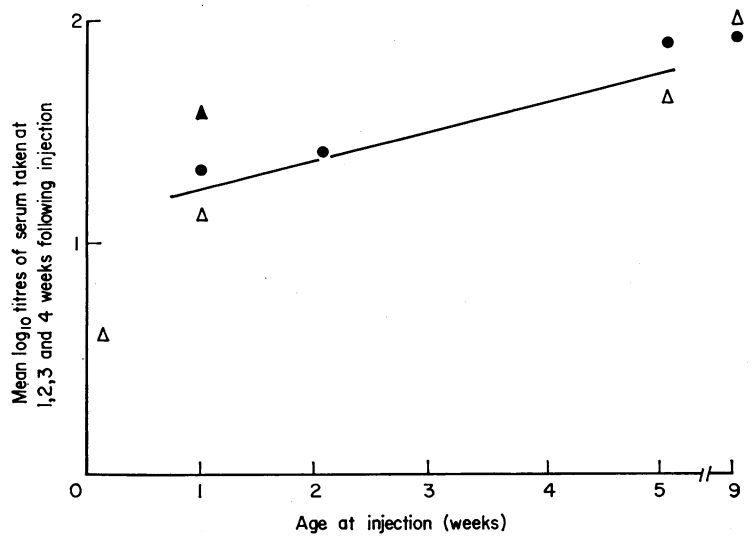

FIG. 3. Primary agglutinin response to killed *Salmonella gallinarum* in developing chickens. 10^8 (△) and 10^9 (▲) virulent strain, 9240; 10^8 avirulent strain, 9S (●).

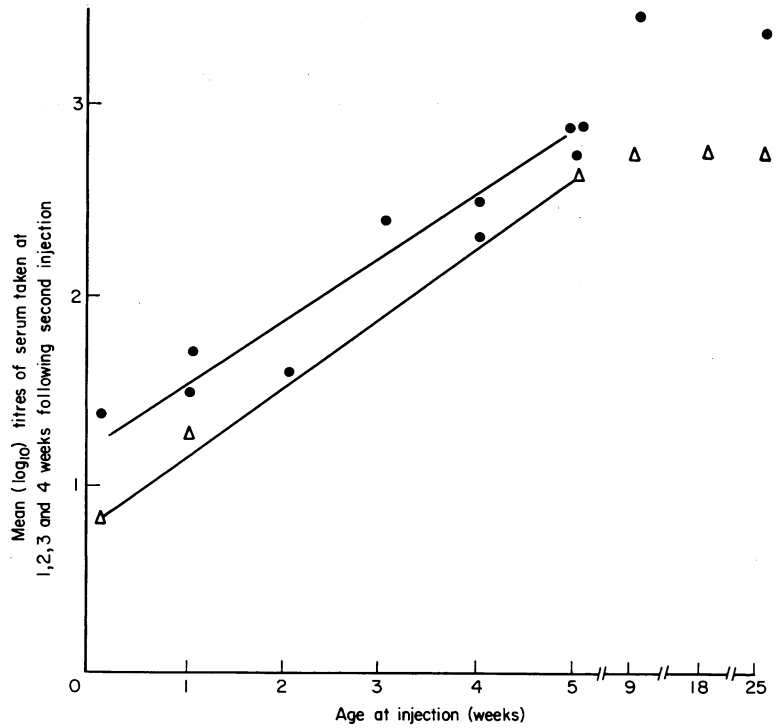

FIG. 4. Primary agglutinin response to live *Salmonella gallinarum* in developing chickens. Virulent strain, 9240 (△); avirulent strain, 9S (●). Doses of bacteria are given in Tables 2 and 3.

of these agglutinins has not been investigated; previous studies suggest that such antibodies could be of a highly heterogeneous character (Delhanty and Solomon, 1966).

CHARACTERISTICS OF THE PRIMARY AND SECONDARY RESPONSES IN ONTOGENY

Peak agglutinin titres of both responses occurred at 7 days after either primary or secondary injection (except for primary responses of 1-day- and 1-week-old chicks). The primary responses of 1 day, 1- and 2-week-old chickens slowly rose to a peak at 2–3 weeks after injection. Responses of 3- and 4-week-old birds rose more rapidly to form a plateau from 1 to 4 weeks after injection (Fig. 2). Five- and 25-week-old chickens showed similar rates of increase during the 1st week after injection, which were more rapid than those for younger birds. Final titres of the primary response at 4 weeks after injection increased with age. As the secondary response increased at the same rate for all ages (except 1-day-old chicks), the peak titres at 1 week after injection also increased with age (Fig. 2).

ONTOGENY OF THE PRIMARY RESPONSE

Killed bacteria (Fig. 3) induced a weaker response than live bacteria in 5- and 9-week-old chickens (Fig. 4). There was an exponential increase in the mean titre of the primary response to live bacteria during the first 5 weeks of age (Fig. 4; Tables 2 and 3). The

TABLE 2

PRIMARY AND SECONDARY RESPONSES TO LIVE AND KILLED VIRULENT STRAIN, 9240, OF *Salmonella gallinarum* DURING DEVELOPMENT

Age at first injection	First dose of bacteria	Reciprocal agglutinin titres	
		Primary response*	Secondary response†
1 day	110 live	6	64
	10^8 killed	4	128
1 week	200 live	20	80
	10^8 killed	14	—
	10^9 killed	38	—
2 weeks	500 live	28	2048
5 weeks	10^8 live	454	2200
	10^8 killed	45	2780
9 weeks	10^8 live	606	—
	10^8 killed	98	—
18 weeks	10^9 live	608	1626
25 weeks	10^8 live	608	3260

* Mean titre of sera taken at 1, 2, 3 and 4 weeks after injection.
† Mean titre of sera taken at 1, 2 and 3 weeks after injection.

avirulent strain (9S) generally elicited slightly higher titres than the virulent strain. Reciprocal titres in normal chicken increased from <4 at 1 day and 1 week, to 8 at 5 weeks and 16 at 25 weeks of age.

ONTOGENY OF THE SECONDARY RESPONSE

Priming occurred from 2 weeks of age with both strains of live *S. gallinarum* (Fig. 5; Tables 2 and 3). The amount of priming (area above the mean titres of the respective

TABLE 3

PRIMARY AND SECONDARY RESPONSES TO LIVE AND KILLED AVIRULENT STRAIN, 9S, OF *Salmonella gallinarum* DURING DEVELOPMENT

Age at first injection	First dose of bacteria	Reciprocal agglutinin titres	
		Primary response*	Secondary response†
1 day	10^3 live	24	120
1 week	10^8 live	32	256
	10^9 live	50	128
	10^8 killed	20	256
2 weeks	10^8 live	38	2560
	10^8 live	—	2046
	10^8 killed	22	644
3 weeks	10^8 live	256	2980
4 weeks	10^8 live	304	—
	10^8 live	215	4080
5 weeks	10^8 live	860 (2)	5150
	10^9 live	608	—
	10^{10} live	304	1626
	10^8 killed	84	5160
9 weeks	10^8 live	3060	—
	10^8 killed	90	—
25 weeks	5×10^8 live	2720	6500

* Mean titre of sera taken at 1, 2, 3 and 4 weeks after injection.
† Mean titre of sera taken at 1, 2 and 3 weeks after injection.

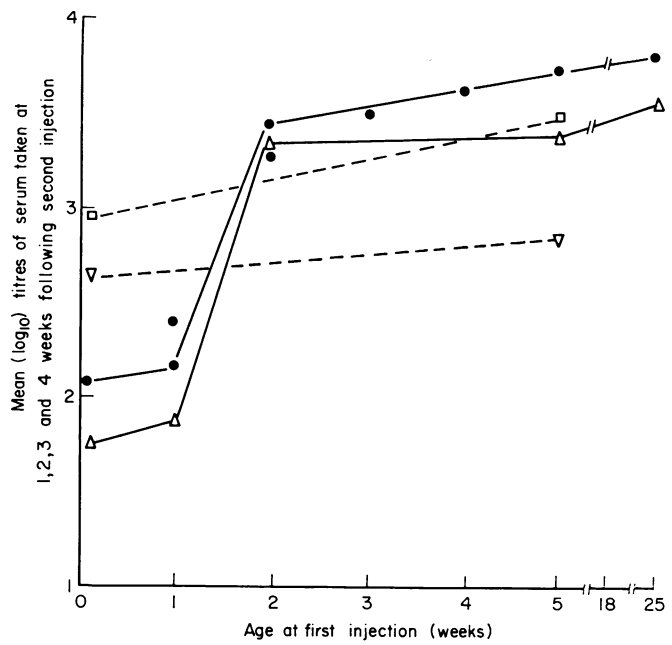

FIG. 5. Induction of tolerance (depressed response) or priming (enhanced response) by live *Salmonella gallinarum*, virulent strain, 9240 (△), avirulent strain, 9S (●) tested by second injection. Doses for the first injection—see Tables 2 and 3. Dose at second injection was 10^8 live bacteria. Controls consisted of the primary response to 9240 (▽) or 9S (□) in normal chickens of the same age as the experimental group at the time of the test injection.

primary responses) was greater with the virulent strain. Killed bacteria primed to an increasing extent from 1 to 5 weeks of age (Fig. 6).

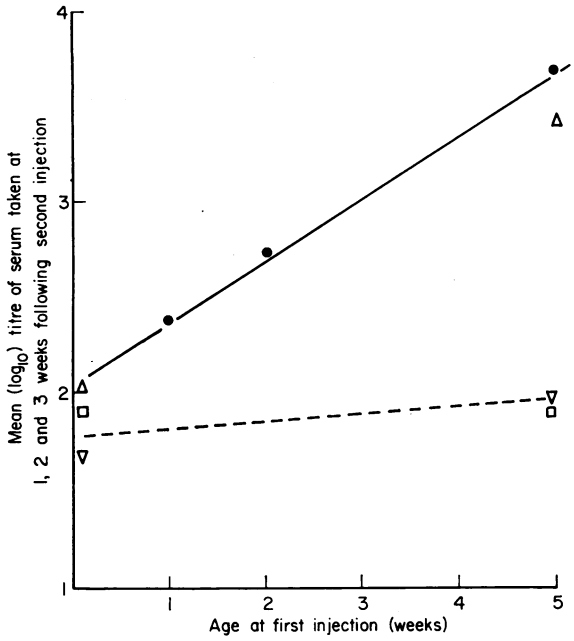

FIG. 6. Induction of priming (enhanced response) by killed *Salmonella gallinarum*, virulent strain, 9240 (△), avirulent strain, 9S (●) tested by second injection. Both injections were 10^8 killed bacteria. Controls consisted of the primary response to 9240 (▽) or 9S (□) in normal chickens of the same age as the experimental group at the time of the test injection.

THE INDUCTION OF IMMUNOLOGICAL TOLERANCE

When 15-day-old embryos were injected with heat-killed *S. gallinarum* there was no increase in mortality at hatching, but 5 weeks later only three out of nine were alive. The other died within 1 week after challenge with *S. gallinarum*. Embryos similarly injected with *S. pullorum* also hatched well, but 5 weeks later only seven out of thirteen

TABLE 4

INDUCTION OF IMMUNOLOGICAL TOLERANCE TO SOMATIC ANTIGENS O-9 AND O-12 IN 15-DAY-OLD CHICK EMBRYOS

Weeks after challenge with 1×10^8 *Salmonella gallinarum* 9S	Reciprocal agglutinin titres	
	Control*	Experimental†
1	4096	16
2	1024	256
3	512	256
4	256	512

* Normal 5 week-old chickens.

† 5-week-old chickens previously injected with $2{\cdot}5 \times 10^5$ *S. pullorum* at 15 days of incubation.

survived. The survivors and a group of normal birds of the same age were injected with 1×10^8 live *S. gallinarum* and bled at 7-day intervals for the following 4 weeks. All the control group survived, but three more birds in the experimental group died during this period. The survivors in the group which had received an injection of *S. pullorum* in embryonic life had significantly depressed agglutinin titres (Table 4).

The subsequent secondary response was significantly suppressed when 110 live organisms of the virulent strain or 1×10^3 of the avirulent strain were injected into 1-day-old chicks. A similar degree of tolerance was also induced by large doses (10^9) bacteria of the avirulent strain in 1-week-old chicks (Fig. 6; Tables 2 and 3). No tolerance was induced in 1-day- to 5-week-old chickens with 10^8 or 10^9 killed bacteria (Fig. 6).

POSSIBLE INHIBITION OF BURSAL GROWTH BY BACTERIA

Tolerance to live bacteria may be due to a 'chemical bursectomy' by lipopolysaccharides derived from bacteria after their death in the host. However, the injection of eleven 1-day-old chicks with 1×10^3 live *S. gallinarum* 9S did not affect the growth of the bursa (Table 5).

TABLE 5

ABSENCE OF INHIBITION OF BURSAL GROWTH AFTER A TOLEROGENIC DOSE OF LIVE *Salmonella gallinarum* 9S

	Bursa weight (mg)/body weight (g)	
	At 3 weeks old	At 5 weeks old
Control	2·8	2·9
Experimental	3·3	3·0

INDUCTION OF IMMUNOLOGICAL TOLERANCE AND SUSCEPTIBILITY TO INFECTION

The onset of resistance to infection with the avirulent strain of *S. gallinarum* occurs between 1 and 5 days after hatching (Solomon, 1968a). This onset of resistance cannot be correlated with ability to form agglutinins because only low titres (1:8 and 1:16 to the virulent and avirulent strains, respectively) were elicited at 1 week after injection of 1-day, 1- and 2-week-old chickens (Fig. 7). The 1st week after injection is critical, because the majority of birds die at 4–5 days after injection with a lethal dose of *S. gallinarum*. Moreover, chickens partially tolerant to at least the somatic O-9,12 antigens of *S. gallinarum* are no more susceptible to infection than normal chickens. A group of thirty 1-day-old chicks were injected with fifty live virulent strain; no deaths occurred when they were challenged with either 1×10^8 virulent strain at 6 weeks or with 1×10^9 at 5 weeks of age.

DISCUSSION

It has been suggested that a higher degree of immunity is required to overcome the attack of the virulent strain of *Salmonella gallinarum*, which has a much greater ability to multiply in young chicken than the avirulent mutant. At 1 week of age the chicken is still susceptible to small doses of the virulent strain and resistance to infection increases

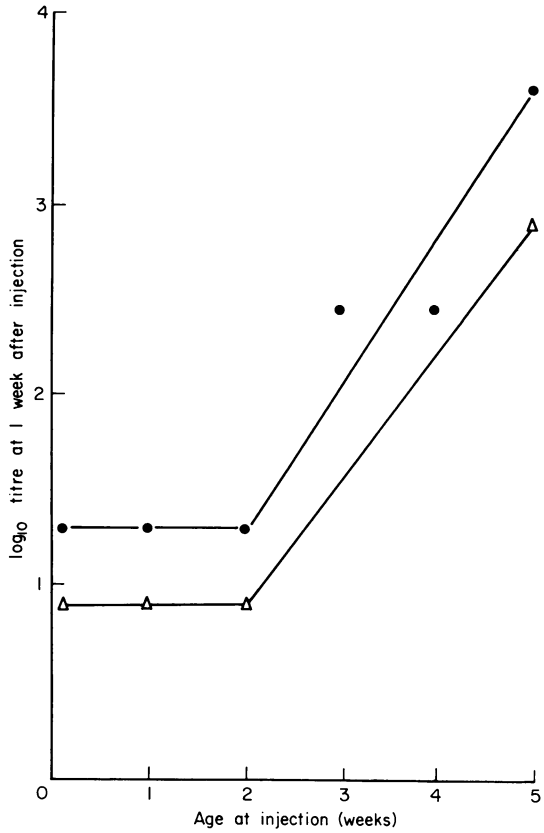

FIG. 7. Agglutinin titres at 1 week after first injection of bacteria during development. Virulent strain, 9240 (△); avirulent strain 9S (●). Doses are given in Tables 2 and 3.

slowly during 1–5 weeks of age (Solomon, 1968a). Agglutinin titres (at 1 week after injection) increase from 2 to 5 weeks of age (see also Prince and Garren, 1966), indicating that the ability to form antibody increases until chicken are resistant. In contrast, resistance to the avirulent strain matures rapidly during the first 5 days of age. The avirulent mutant has a diminished ability to multiply in the host and therefore even a low level of immunological competence will prevent growth to population levels which are lethal for the host. The onset of resistance to pneumococci (Andrews and McKinnon, 1961) and *S. pullorum* (Severens, Roberts and Card, 1944) also increases rapidly during the first 5 days after hatching; this coincides with the onset of antibody formation to erythrocyte antigens (Solomon and Tucker, 1963).

Previously, priming of cells for the secondary response (with bovine serum albumin) has only been demonstrated in chickens of at least 6 weeks of age (Blazkovec and Wolfe, 1965). Killed or live bacteria, on the other hand, will prime as early as 1 or 2 weeks after hatching, respectively. However, killed bacteria did not induce as great a primary response in 5-week-old chicken as live *S. gallinarum*. In addition, killed bacteria did not prime younger birds as effectively as live organisms. There was little increase in the number of cells primed by live bacteria during development, which is in accord with Makinodan

and Allbright's (1967) thesis that antigen primes the same average number of additional cells throughout development.

A decrease in the weight of the bursa of Fabricius and involution of its lymphoid follicles has been observed in acute infections of White Leghorns with *S. gallinarum* (Garren and Barber, 1955). This suggested that bacteria might effect a degree of 'chemical bursectomy' which could possibly account for the induction of tolerance to bacteria, since it has been shown that surgical bursectomy, soon after hatching, results in increased susceptibility to *S. typhi* (Chang, Rheins and Winter, 1959), *S. typhimurium* and *Spirochaeta gallinarum* (Perek and Drill, 1962). However, no inhibitory effects on the growth of the bursa were observed in the present experiments.

Partial tolerance has been induced in chick embryos to soluble *Shigella* antigen (Friedmand and Gaby, 1960) diphtheria toxoid (Gowland and Oakley, 1960) and killed *Salmonella pullorum* (Buxton, 1954). The work of Buxton has been confirmed using *S. gallinarum* for the challenge injection (both strains share identical major groups of somatic-O antigens). There are few examples of successful induction of tolerance to live bacteria in neonates and these have all required a large number of injections. Such experiments include the induction of partial tolerance to *S. paratyphi B* in neonatal rabbits (Šterzl and Trnka, 1957), to *Brucella abortus* in lambs (Nagy, 1963) and calves (Hignett and Nagy, 1964) and to *Pseudomonas* sp. in neonatal rabbits (Gowland, Hobbs and Byers, 1965).

The dosage-dependence of the induction of tolerance to *Salmonella gallinarum* is difficult to assess. Small doses of killed bacteria will induce tolerance in embryos, yet large doses only induce immunity in hatched chicken. Multiplication of bacteria in the liver and spleen does not appear to be essential for the induction of tolerance. For example, tolerance may be induced with 10^9 organisms of the avirulent strain in 1-week-old chickens, which are naturally resistant to infection with this strain and which rapidly kill such organisms (Solomon, 1968a). Apart from this case, tolerance was induced in those conditions where the host was susceptible to infection and bacteria multiplied in the liver and spleen for a few days after injection. It is not possible to predict whether the small numbers of bacteria used to induce tolerance do so immediately (low-dose induction, Mitchison, 1964) or whether the larger populations of bacteria, derived from growth of the bacterial inoculum, constitute the tolerogenic stimulus. The virulent strain of *S. gallinarum* does not elicit agglutinating antibody quite so readily as the avirulent strain and, therefore, might be expected to be a better tolerogen. Such factors, coupled with its greater proliferative ability, may explain why so few organisms of the virulent strain will induce tolerance. Similarly, the extent of proliferation of foreign cells in the graft against the host reaction has been tentatively equated with the degree of induction of tolerance (Solomon, 1962).

Chickens are susceptible to infection with the virulent strain until 5 weeks of age, yet the appearance of cells capable of being primed from 2 weeks of age precludes any possibility of testing whether the majority of cells initially responded to antigen by becoming tolerant. However, it seems that cells producing agglutinins are not those directly concerned with protective mechanisms to *S. gallinarum* infection. Therefore, tolerance cannot be closely associated with susceptibility to infection. For example, chickens which are resistant to a challenge injection of the virulent strain can be immunologically unresponsive with respect to agglutinin formation. Again, 1-week-old chicks, which are highly resistant to the avirulent strain, may be made tolerant with large doses of this strain. These findings suggest that cells, which are responsible for the production of agglutinating

antibody, appear to be independent of other members of the same clone which furnish some type of protective immunity.

Although resistance to *S. gallinarum* infection may be induced by injection of the live avirulent mutant (Smith, 1956), even high titres of agglutinins to the somatic O-9,12 antigens do not afford protection (Buxton and Field, 1959). Also, killed *S. gallinarum* does not elicit protective immunity, yet it will sometimes induce the formation of agglutinins just as effectively as live bacteria. As the nature of resistance to *S. gallinarum* infection has not yet been clearly identified with any known type of antibody (Buxton and Field, 1959; Solomon, 1968a, b, c), immunological tolerance cannot be equated with susceptibility to infection until the nature of the protective antibody is known.

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