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Effect of Specific Immune Mouse Serum on the Growth of *Salmonella enteritidis* in Mice Preimmunized With Living or Ethyl Alcohol-killed Vaccines

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The effect of prior opsonization of virulent *Salmonella enteritidis* on the growth of this organism in blood, liver, spleen, peritoneal cavity, and inguinal lymph node of specific pathogen-free mice prevaccinated with ethyl alcohol-killed *S. enteritidis* or living *S. gallinarum* was determined by daily enumeration. Both the vaccines and the challenge inocula were injected by the intravenous, intraperitoneal, or subcutaneous routes to determine the effect of variations in the vaccinating procedure on the level of immunity induced. The survival percentage observed in mice vaccinated with killed organisms varied extensively, depending on the route of challenge. However, simultaneous organ enumeration studies revealed that vaccination with killed organisms failed to prevent the growth of the challenge organism in vivo. On the other hand, virulent *S. enteritidis* injected into mice vaccinated with living *S. gallinarum* failed to multiply and was subsequently eliminated. Immunity in these animals was so effective that a subcutaneously injected challenge did not spread beyond the regional node. Immunization with killed organisms slowed but was unable to prevent the spread of such a challenge beyond the draining node involved in the primary immune response. Neither the route of challenge nor the regimen used in the vaccination had any appreciable influence on the level of antibacterial immunity detected in the organs of the reticuloendothelial system at the time of challenge.

Salmonella enteritidis is a facultative intracellular parasite able to proliferate freely in an intracellular environment (22). Specific immune serum promotes the phagocytosis of this organism (16), but once this process is complete, those bacteria capable of surviving the initial period of inactivation are free to multiply within their intracellular environment, protected against further exposure to opsonic or bactericidal antibody (27).

In the preceding study (6), attempts to verify claims for passive serum protection (15, 17, 28, 29) against intravenous, intraperitoneal, or subcutaneous challenge with *S. enteritidis* were uniformly unsuccessful if immunity was assessed in terms of the behavior of the pathogen in the liver, spleen, and regional lymph node of the challenged mice. Examination of such enumeration data suggested that those mice that survived the challenge did so by virture of a resistance mechanism developed in response to the infection itself (4, 6, 7). It could be argued, however, that the inability of immune serum to suppress the spread of the infection in the recipient was due to the rapid elimination of the passively administered protective antibodies rather than to their initial absence from the hyperimmune serum. To answer this objection, the present studies were carried out using actively immunized mice in which the availability of antibody would no longer be critical. At the same time, comparison was made between these data and those obtained from protection experiments carried out in mice immunized with a living vaccine. The results show that antibody alone does not provide a basis for the mechanism by which the host is able to effectively eliminate an infecting population of virulent salmonellae, irrespective of the route of vaccination or challenge.

MATERIALS AND METHODS

Organisms. *S. enteritidis* 5694 and *S. gallinarum* 9240 have been described previously (4, 7).

Animals. Specific pathogen-free COBS mice (Charles River Farms, Inc.) were maintained under conditions previously described (8). Eight-week-old

females were used throughout. The mice receiving killed vaccines were maintained under isocaps (Carworth-Lab Cages, New York City, N. Y.).

Vaccination of mice with living *S. gallinarum* or ethyl alcohol-killed *S. enteritidis* was carried out as described elsewhere (8). Sterility tests were made on the killed vaccines and on homogenates of the livers and spleens of the vaccinated mice as described earlier (7). The routes of injection and the size and the number of doses of killed vaccine are indicated in the text.

Serology. The methods used were those described in the previous paper (6).

Opsonization of S. enteritidis challenge. *S. enteritidis* 5694 was opsonized with specific hyperimmune mouse serum, as described in the preceding paper (6). The opsonized suspension was subjected to sonic vibration for 5 sec to break up any clumps of bacteria. The number of viable bacteria in the challenge dose was checked by plating suitable serial dilutions on digest-agar plates.

Enumeration of the in vivo population. The numbers of bacteria in the blood, liver, spleen, peritoneal cavity, and inguinal lymph node were estimated daily on five randomly selected mice, as described previously (6). In mice vaccinated with living *S. gallinarum*, the liver and spleen populations were double-plated on digest-agar with and without 5 μg of streptomycin per ml. *S. gallinarum*, being resistant to 5 μg of drug per ml, permitted the simultaneous enumeration of both challenge and vaccine populations (23).

RESULTS

Growth of opsonized S. enteritidis in mice vaccinated intraperitoneally with ethyl alcohol-killed S. enteritidis. One hundred mice were injected three times a week for 3 weeks with 10^6 ethyl alcohol-killed *S. enteritidis*. They were divided into two groups and challenged 7 days later with opsonized *S. enteritidis* by the intravenous or intraperitoneal routes, and the resulting growth curves are recorded in Fig. 1. Similar studies employing an unopsonized challenge gave essentially similar results, as might be expected in view of the antibody levels present in these mice (Table 1). The introduction of the challenge population by the same pathway or by a route other than that used for vaccination appeared to have little effect on the fate of the challenged animals compared with that observed in the unvaccinated controls (Table 2).

Vaccine injected subcutaneously. When 150 mice were injected subcutaneously three times a week for 3 weeks with 10^6 ethyl alcohol-killed *S. enteritidis* and challenged 7 days later either intravenously, intraperitoneally, or subcutaneously, the growth curves shown in Fig. 2 were obtained. The effect of subcutaneous vaccination on the level of resistance to challenge by this same route was followed with especial interest because the primary immune response is presumed to have occurred in the regional lymph node, a circumstance which Kenny and Herzberg (20) regard of potential importance. After subcutaneous vaccination, the inguinal lymph node was grossly enlarged, and the time taken for the subcutaneously injected challenge to reach the liver and spleen was delayed for 4 to 5 days (Fig. 2). Whereas subcutaneous vaccination protected most of the mice from death (Table 2), it failed to prevent the spread and subsequent growth of the organisms in the liver and spleen (Fig. 2). Presumably, the increased survival was, as in previous studies (7, 9), attributable to the resistance engendered by the challenge infection and not to that which resulted from the vaccination, which merely delayed the spread of organisms to liver and spleen but could not subsequently influence their rate of growth.

Killed vaccine simultaneously injected intravenously, intraperitoneally, and subcutaneously. One hundred fifty mice were injected three times weekly for 3 weeks with 10^6 ethyl alcohol-killed *S. enteritidis* by each of the three routes. The mice were rested for 7 days and then divided into three groups. Each group was challenged intravenously, intraperitoneally, or subcutaneously with 100 to 1,000 LD_{50} of opsonized *S. enteritidis*; the growth curves so obtained are recorded in Fig. 3. The survival percentages for the three groups of mice (Table 2) indicate that a significantly increased level of protection was present in mice immunized with the killed vaccine and then challenged subcutaneously, but not when the organism was introduced intravenously or intraperitoneally. However, the corresponding growth curves obtained from these mice (Fig. 3) clearly demonstrated the absence of any antibacterial immunity in any of the mice, judging from the extensive in vivo growth observed in all three groups of animals.

Growth of opsonized S. enteritidis in mice vaccinated intravenously with living S. gallinarum. Although earlier studies demonstrated the presence of an effective immunity against intravenous challenge by *S. enteritidis* (7, 8), this protection experiment was repeated to provide a quantitative comparison with mice challenged by the other two routes. Furthermore, past studies have employed only unopsonized bacteria for the challenge; therefore, the effect of preopsonization on the fate of the challenge had to be determined.

When mice were vaccinated intravenously with 0.5 LD_{50} of living *S. gallinarum* (approximately 2.0×10^6 organisms), a liver and spleen growth curve similar to that reported previously (7, 8) was obtained. When these mice were challenged on day 8 with opsonized *S. enteritidis* injected by

either the intravenous, intraperitoneal, or subcutaneous routes, the growth curves shown in Fig. 4 were obtained. As expected, the fate of an intravenous challenge by opsonized *S. enteritidis* in *S. gallinarum*-vaccinated mice was no different from that previously reported for unopsonized bacteria (7). The survival figures shown in Table 2 also attest to the protective value of the living,

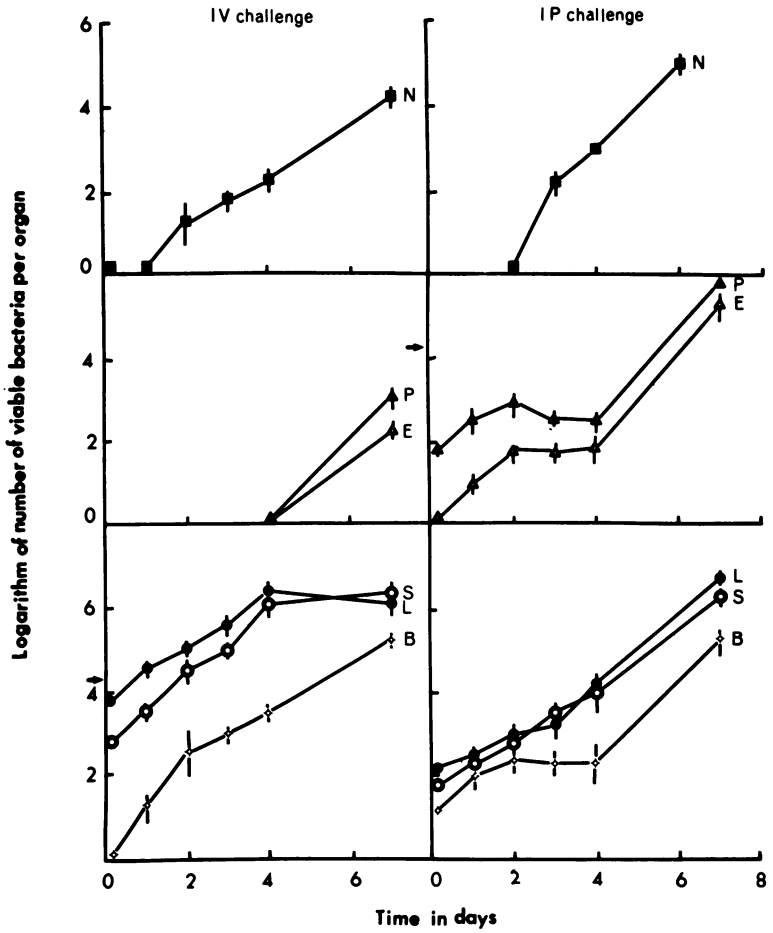

FIG. 1. *Growth of opsonized S. enteritidis 5694 in mice preinmmunized with intraperitoneally injected ethyl alcohol-killed S. enteritidis after intravenous (left) or intraperitoneal (right) challenge. Abbreviations: L, liver; S, spleen; B, blood; P, peritoneal population (intracellular); E, extracellular peritoneal bacteria; and N, inguinal lymph node. The size of the challenge inoculum is represented by the arrow head. Standard error is represented by vertical lines drawn through each time point.*

TABLE 1. *Agglutinating, hemagglutinating, and bactericidal antibody titers in vaccinated mouse sera*

Vaccine	H agglutinating antibody	O agglutinating antibody	Hemagglutinating vs. *S. enteritidis* LP	Bactericidal antibody
Ethyl alcohol-killed *S. enteritidis*	160[a]	40	2,560	10^4
Living *S. gallinarum*	<10	10	160	—[b]
Living *S. enteritidis*	20	10	320	10^3
None	<10	<10	<10	$<10^2$

[a] Inverse of titer.
[b] Not tested.

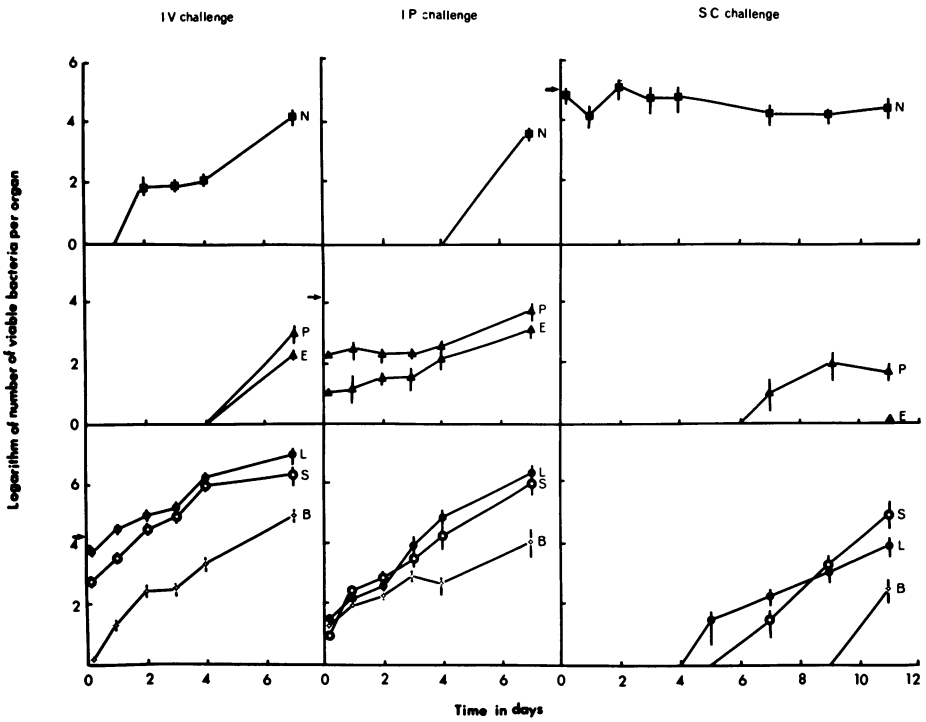

FIG. 2. *Growth of opsonized S enteritidis 5694 in mice preimmunized with subcutaneously injected, ethyl alcohol-killed S. enteritidis after intravenous (left), intraperitoneal (middle), or subcutaneous (right) challenge. See the legend to Fig. 1 for the key to the curves.*

attenuated vaccine. In fact, an effective antibacterial immunity was easily demonstrable in all groups of mice irrespective of the route of challenge employed (Fig. 4 and Table 2). Although, in both the intraperitoneally and subcutaneously challenged mice, the population remaining at the site of infection declined relatively slowly, there was an absence of the extensive and rapid growth invariably observed in the livers and spleens of mice vaccinated with a killed vaccine (compare Fig. 3 and 4).

Subcutaneously vaccinated mice. The actual route of vaccination employed did not appear to be critical because an equivalent level of antibacterial immunity was also observed in mice vaccinated subcutaneously with living *S. gallinarum* 10 days previously (Fig. 5).

DISCUSSION

When mice immunized with living or dead vaccines are challenged by the intravenous route, the superiority of the living vaccine is clearly demonstrable whether the criterion of immunity is based upon overall mortality (18, 24, 31, 32) or the growth pattern of the challenge organism in vivo (7, 8, 13, 33). This, however, is not true for the subcutaneous or the intraperitoneal routes of challenge. Animals vaccinated with killed organisms can frequently survive challenge, but viable counts reveal that the challenge organism multiplies almost as extensively in them as it does in normal controls (3, 9, 12, 13, 33). It cannot be denied that vaccination with dead organisms results in an increased survival rate, but the reason for this is only indirectly related to changes induced by the vaccine itself. In animals immunized with living organisms, on the other hand, the challenge organism is eliminated from the tissues usually without multiplying and without any necessity on the part of the host to mount a response which will result in the development of that type of cellular immunity necessary for the control of an infection by a facultative intracellular parasite such as *S. enteritidis* (5, 8). Vaccination with heat, ethyl alcohol- or radiation-killed organisms (14, 20, 26), or extracts thereof (9, 17, 19), will usually result in the survival of a proportion of the treated mice. The antibodies so induced protect first by reducing the size of the initial viable population and then by slowing the rate of dissemination of the survivors from their subcutaneous or intraperitoneal sites of implanta-

TABLE 2. *Progressive mortality in mice vaccinated with ethyl alcohol-killed S. enteritidis or with living S. gallinarum and then challenged with S. enteritidis by different routes*[a]

Vaccine	Vaccination route[b]	Challenge route	No. of deaths on day										
			5	7	8	9	10	11	12	13	14	21	28[c]
Ethyl alcohol-killed *S. enteritidis* (3×10^6)	IP	IV	0	1	1	2	4	4	5	7	8	10	10/10
	IP	IP	0	1	2	2	3	4	4	6	6	6	6/10
	IP	SC	0	0	0	0	0	0	0	2	2	4	4/10
	SC	IV	1	3	3	4	4	4	6	6	7	9	9/10
	SC	IP	0	0	1	2	2	3	5	6	7	7	7/10
	SC	SC	0	0	0	0	0	0	0	0	1	2	2/10
	IV, IP, SC	IV	0	2	3	3	3	3	5	5	6	6	6/10
	IV, IP, SC	IP	0	1	1	2	4	7	7	7	8	8	8/10
(9×10^6)	IV, IP, SC	SC	0	0	0	0	0	0	0	0	1	2	2/10
Control		IV	2	4	4	6	6	6	6	9	9	10	10/10
		IP	0	1	2	4	4	4	7	8	9	9	9/10
		SC	0	0	0	0	0	0	0	1	1	2	5/10

[a] There were no deaths among mice vaccinated with living *S. gallinarum* (2×10^6) and challenged with *S. enteritidis*.

[b] IV, intravenous; IP, intraperitoneal; SC, subcutaneous.

[c] Deaths/total number of mice.

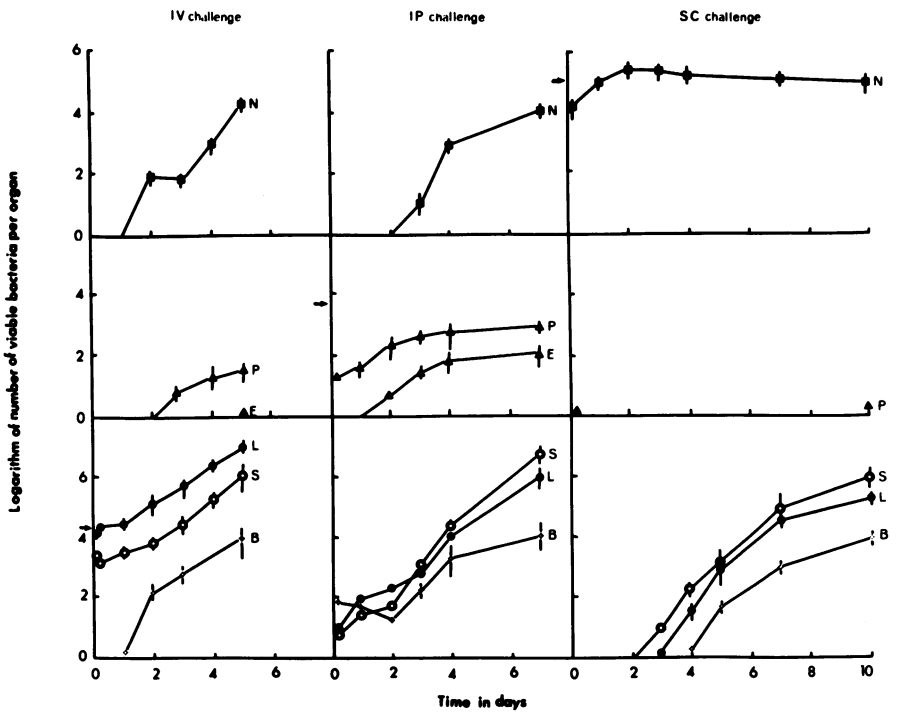

FIG. 3. *Growth of opsonized S. enteritidis 5694 in mice immunized with ethyl alcohol-killed S. enteritidis injected simultaneously by the intravenous, intraperitoneal, and subcutaneous routes and then challenged by the intravenous (left), intraperitoneal (middle), or subcutaneous (right) routes. See the legend to Fig. 1 for the key to the curves.*

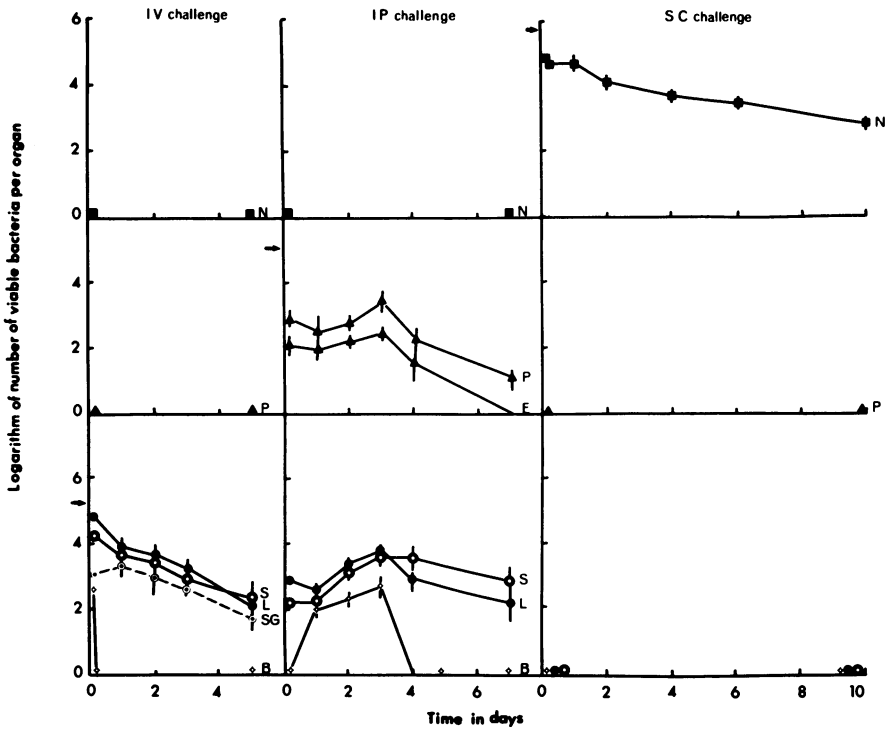

FIG. 4. *Growth of opsonized S. enteritidis 5694 in mice vaccinated intravenously with 0.5 LD_{50} of S. gallinarum 8 days previously. The challenge was injected by the intravenous (left), intraperitoneal (middle), or subcutaneous (right) routes. See the legend to Fig. 1 for the key to the curves. The broken line represents the residual S. gallinarum population.*

tion. However, in our experience, they are quite unable to *prevent* this dissemination to the liver, spleen, and elsewhere; they have little, if any, discernible influence upon the subsequent rate of multiplication of the parasite in these organs. Unquestionably, such a reduced rate of dissemination of organisms from the primary site of implantation in mice vaccinated with dead organisms or protected with immune serum is important to the ultimate survival of the host. However, such pretreatment does not, of itself, bring about the elimination of the challenge but merely provides the host with the time required to develop that type of immune response which animals vaccinated with living attenuated organisms are able to exhibit throughout the challenge period (7, 13, 23, 33). Such a response results in the activation of a mechanism of resistance which is superior to that obtained with dead vaccines, since it is capable not only of preventing the early multiplication of the challenge organisms but also of subsequently bringing about their complete elimination (8).

Much of the present confusion over the use of the term "protective" in relation to antigenic preparations, and the role of the corresponding immunoglobulins in the resulting immunity they mediate, stems from the fact that these terms mean different things to different workers. The mere survival of a statistically significant proportion of a challenged population (16) overlooks the fact that most of the survivors will have undergone a severe, nearly fatal, clinical infection. When attention has been paid to this point [as in the case of human antityphoid trials (2)], the protective value of nonliving vaccines has been shown to be barely significant (10, 34). In this laboratory, only those vaccination procedures which generate a fully effective antibacterial immunity capable of eliminating a lethal dose of a highly virulent organism without the development of the clinical disease have been regarded as "protective" (3, 4, 7, 8, 9, 23). The tendency to ascribe the increased survival of vaccinated animals to the immediate influence of immunization on the level of immunity at the time of challenge (14, 15, 17, 29) is a dangerous oversimplification which ignores the

fact that the challenge infection provokes its own immune response and is the ultimate cause of the survival of the host (21, 22).

Work from this laboratory has concentrated attention upon those aspects of the host's response to infection which are responsible for eliminating the pathogenic salmonellae from the tissues (5, 8). In the ultimate analysis, the mechanism by which the host finally achieves this end must be considered to constitute the essential mechanism of acquired resistance. All of the indications are that this depends upon a modification of the functional status of host phagocytes (1, 21, 22, 25) rather than on the production of humoral antibody (27). This conclusion was reached on the basis of in vivo studies in which the intravenous route of challenge was used (7, 23) and on parallel studies in which the functional status of the cells of the reticuloendothelial system was evaluated (1, 3, 33). It has been objected, however, that the intravenous route of challenge is so unlike the natural route of infection that the results obtained do not faithfully reflect the true state of host resistance after vaccination (20). It has been argued that infection by the natural portal of entry through the gut inevitably involves lymphatic organs early in the dissemination of infection (27, 30), and that subcutaneous or intraperitoneal routes of challenge are, therefore, more appropriate. Since killed vaccines do provide a measure of protection against challenge by these routes (17, 20, 28), it has been concluded that humoral antibody, which is also claimed to be capable of providing passive protection against challenge by these routes (28, 29, 32), is the basic mechanism of acquired immunity against virulent salmonella infections in the mouse (16). Because no evidence for this could be obtained in animals challenged by the intravenous route (4, 7, 9, 12, 13), these studies were repeated using the same method of bacterial enumeration in an effort to determine whether killed vaccines or immune serum can, in fact, be more effective against an intraperitoneal or subcutaneous challenge than they are against intravenous challenge. The results of these studies were, however, comparable in every respect to those reported previously (6, 8).

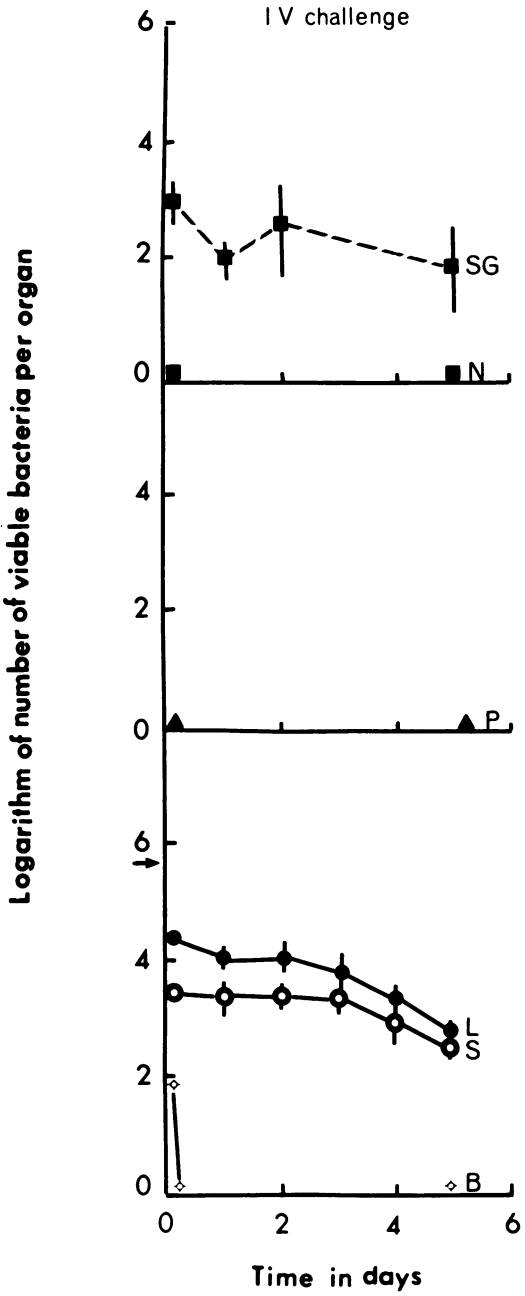

FIG. 5. *Growth of an intravenous challenge by opsonized S. enteritidis 5694 in mice vaccinated 10 days previously by the subcutaneous route using 10^6 living S. gallinarum. See the legend to Fig. 1 for the key to the curves.*

The ultimate objective of any study of immunoprophylaxis is to find a vaccine that does not merely protect a proportion of the animals against a virulent challenge introduced by one selected pathway but provides the mice with augmented defenses sufficient to completely prevent the development of the disease, irrespective of the route of challenge. Vaccinating procedures that bring about the production of high levels of humoral antibody do not appear to satisfy these requirements. In this study, saline suspensions of killed *S. enteritidis* produced levels of opsonic and bactericidal antibodies comparable to those reported by Kenny and Herzberg (20) and others (11, 12, 30, 31). They did not, however, prevent the establishment of the infection in any of the

mice examined, despite the improved survival rates.

As previously reported (8), saline suspensions of killed salmonellae also fail to induce the state of delayed-type hypersensitivity which invariably develops in animals actively infected with either virulent or attenuated organisms. Moreover, only those strains of salmonellae which can survive in vivo are capable of inducing this type of hypersensitivity and of providing complete protection against the virulent challenge (4, 5, 7, 8). Herein, perhaps, lies the essential difference between the immunizing potentials of living and dead vaccines. Therefore, future studies of this problem might be more profitably centered on a search for those conditions of immunization which lead to the production of delayed-type hypersensitivity rather than humoral antibody. It is in this way, perhaps, that a method of immunization with dead bacteria can be found that is equal in its efficacy to that provided by a living vaccine.

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