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Effect of Iron on Antibacterial Immunity in Vaccinated Mice

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The effect of iron on resistance to *Salmonella typhimurium* was investigated in mice inoculated with vaccines prepared from live and avirulent (SL3770) or killed and virulent (SR11 or LT2) bacteria. It has been found that mice vaccinated with SL3770 vaccine develop an immunity which can be neutralized with iron. Iron promoted the development of lethal infections by serving as a growth-essential nutrilite for infecting bacteria and by neutralizing the acquired immunity. The titration of this dual effect of iron showed that more iron was needed to neutralize the immunity in vaccinated animals than to promote bacterial growth in normal animals. In the presence of a sufficient amount of exogenous iron, as few as 10 bacteria caused lethal infections in normal and immune mice with the same effectiveness. This iron-sensitive immunity could be changed to iron-resistant immunity by the immunological stimulation of SL3770-vaccinated mice with a sonicated vaccine prepared from heat-killed SR11 or LT2 bacteria. In distinction to iron-sensitive immunity, iron-resistant immunity could be transferred from SR11- or LT2-stimulated to normal mice with serum. Although effective in the transfer of antibacterial immunity, sera of SR11- or LT2-stimulated mice supported the growth of virulent bacteria as well as did sera of normal mice. The absorption of immune serum with either SR11 or LT2 bacteria removed its protective quality, but the sensitized bacteria remained as infectious as untreated bacteria for iron-treated normal mice. Only in SL3770-vaccinated mice were the immune serum-sensitized bacteria not able to cause the infection in spite of daily treatment with iron. These results suggest that iron-resistant immunity is due to the synergistic action of specific antibody and phagocytes of immunologically stimulated animals.

In response to microbial infections, animals develop nutritional immunity which helps them to suppress microbial growth by limiting the availability of iron (13, 14). The amount of iron available for use by invading parasites is lowered by the activity or iron-binding proteins, by the development of hypoferremia, by the decreased absorption of iron from the gastrointestinal tract, and by suppression of the production of siderophores or their activity (28). The injection of iron into an infected animal promotes microbial multiplication and induces the development of lethal infections (3, 11, 15).

The ability of virulent bacteria to overcome the iron limitation and to grow in animals or in their sera is being attributed to either the iron-recruiting activity of extracellular siderophores (25, 30) or the cell wall-associated siderophores and lipopolysaccharides (9, 17). Recent results suggest that bacteria obtain iron directly from transferrin with or without participation of siderophores (1, 19). It seems that the acquisition of iron by cell wall-associated factors may play a significant role in bacterial virulence (17, 23).

Investigation of the infection-promoting effect of iron showed that iron predisposes not only normal but also immune animals to bacterial infections (18, 27). Our study of the nature of iron-neutralizable immunity showed that iron does not interfere with the development of immunity and delayed hypersensitivity but does interfere with the expression of antibacterial responses in vaccinated animals (16). We have found, also, that iron does not interfere with the production of antibodies and their serological activities. The investigation described in this report was directed to identify iron-neutralizable immune factors in animals vaccinated with live and killed vaccines of *Salmonella typhimurium*.

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MATERIALS AND METHODS

Bacteria. The effect of iron on infections with virulent *S. typhimurium* was tested in normal and actively or passively immunized mice. Mice were vaccinated with live avirulent SL3770 bacteria or with killed virulent SR11 or LT2 bacteria and infected with virulent bacteria; mean lethal dose values of SR11 or LT2 bacteria and SL3770 bacteria for normal mice were found to be 10^3 and 10^7 cells, respectively (18). Strains of *S. typhimurium* were received from R. J. Roantree; the serotype of LT2 and SL3770 (O4:5:12) is different from that of SR11 (O1:4:12).

Bacterial cultures were maintained with monthly transfer on brain heart infusion agar slants, and bacterial virulence was preserved with passages in mice. Before use in experiments, bacteria were grown in Trypticase soy broth (BBL Microbiology Systems) at 37°C for 18 h. Bacteria were collected by centrifugation and resuspended in physiological saline, and bacterial suspensions were adjusted to a desired density with a Klett colorimeter. Bacterial numbers in various inocula and vaccination or challenge doses were determined by a plating method as described previously (19).

Animals. Female mice of the Swiss-Webster strain, weighing 20 to 25 g each, were used in this investigation. Animals were fed Purina chow and water ad libitum and were housed in temperature- and humidity-controlled rooms. Each experimental group was composed of at least 10 mice.

Immunization and infection. Mice were vaccinated with two intraperitoneal injections of 10^6 live avirulent SL3770 bacteria administered during a 2-week period. Occasionally, strain-specific immunity was induced in vaccinated mice with killed virulent bacteria. In such experiments, 1 week after the second SL3770 vaccination, mice were immunologically stimulated with a sonicated vaccine prepared from 10^8 cells of heat-killed virulent SR11 or LT2 bacteria. Bacteria were killed by exposure to 75°C for 1 h and disrupted by 10

30-s sonical bursts delivered by a microtip at the maximum output of a sonicator (model W225R; Heat Systems-Ultrasonics, Inc., New York, N.Y.). One week after the vaccination period, mice were infected intraperitoneally with 10^3 SR11 or LT2 bacteria. The progression of infection in normal, immune, and immunologically stimulated mice was measured by determining the time and frequency of mortality in LT2- or SR11-infected mice. The bacterial causation of mortality was ensured by determining bacterial numbers in spleen and liver homogenates as described previously (16); both organs of dying mice contained 10^{10} bacteria.

Iron. The effect of iron on development of infections in normal and actively or passively immunized animals was investigated in mice treated daily with an intraperitoneal injection of 0.1 mg of iron. The solution of iron was prepared by dissolving ferric ammonium citrate in saline. The exact amount of solubilized iron was determined colorimetrically by the method of Goodwin and associates (10). Control (iron-untreated) mice were injected daily with 0.56 mg of ammonium citrate present in the solution of ferric ammonium citrate containing 0.1 mg of iron.

Sera. Blood was obtained from normal, immune, and SR11- or LT2-stimulated mice 1 week after inoculation of mice with live and heat-killed vaccine; after the clotting of pooled blood, serum was collected and sterilized by filtration. In the preparation of sera, an effort was made to preserve complement, and in some experiments sera were enriched by the addition of complement-rich guinea pig serum. The effect of sera on bacterial growth was tested in sera adjusted to pH 7.4 and inoculated with 5×10^2 virulent or avirulent bacteria per ml of serum. To determine the effect of iron on the growth of bacteria, some samples of sera were enriched with iron (10 μg/ml). The growth of serum-exposed bacteria at 4, 8, and 12 h was determined by plating diluted samples on nutrient agar and counting bacterial colonies after incubation of inoculated plates at 37°C for 24 h.

Besides use in bacterial growth experiments, normal and immune sera were used in attempts to transfer immunity to normal animals and to sensitize virulent SR11 bacteria with their antibodies. Mice were passively immunized with an intraperitoneal injection of 0.5 ml of a 1:2 dilution of immune serum containing 10^3 SR11 bacteria. The effect of iron on serum-transferred immunity was tested by injecting mice each day with 0.1 mg of iron. In sensitization experiments, 10^8 SR11 bacteria were exposed for 1 h at 37°C to the activity of 1 ml of immune serum; sensitized bacteria were collected by centrifugation, washed with saline, and injected into normal and SL3770-vaccinated mice. In some experiments, immune sera were absorbed twice with SR11 bacteria (10^8 cells per ml), and absorbed sera were used in serum passive transfer experiments.

Serological test. Attempts were made to measure the amount of antibody in immune and immune-activated sera. The sera were tested by the classical Vidal method for their agglutination of heat-killed SR11 bacteria adjusted to 10^9 cells per ml.

Statistical analysis. When appropriate, the significance of data was determined by Student's *t* test. A *P* value of <0.05 was taken as significant.

RESULTS

Iron-sensitive immunity. Injections of iron into SR11-infected normal mice and SL3770-vaccinated mice promoted a rapid development of lethal infections not only in normal but also in immune mice (Fig. 1). During the first 5 days after SR11 challenge, about 75% of iron-treated normal and immune mice died from the infection, and the rate of mortality in both groups was nearly the same. This, as well as the more rapid mortality in iron-treated immune mice than in untreated normal mice ($P < 0.001$), suggested that iron promotes bacterial infection in immune animals not only by serving as an essential nutrilite for infecting bacteria but also by neutralizing the actively acquired immunity. These results support our previous findings which showed that tissues of iron-treated immune mice contain much larger numbers of infecting bacteria than corresponding tissues of untreated normal mice (16).

The strength of antibacterial immunity in SL3770-vaccinated mice and the efficiency with which this immunity can be neutralized with iron were investigated by challenging mice with various numbers of virulent bacteria and by injecting infected mice with various amounts of iron. Results showed that, in contrast to normal mice which succumb to infection with 10^3 to 10^4 bacteria, most of the vaccinated mice were resistant to 10^6 bacteria; the mean lethal infecting dose for SL3770-vaccinated mice was about 1,000-fold higher than that for normal mice. In iron-treated groups, as few as 10 challenge bacteria caused lethal infections in all normal and immune mice. This equal susceptibility of iron-treated normal and immune mice to infection with a few bacteria indicates that antibacterial immunity in vaccinated animals is inactive not because it is overwhelmed by a large number of challenge bacteria, but because it is neutralized by iron.

The possibility that more iron might be required to promote infections in immune than in normal mice was tested in mice infected with 10^3 SR11 bacteria and injected daily with various amounts of iron. Results showed that doses of 50, 25, and 10 μg of iron/mouse per day were less effective in promoting lethal infections in immune than in normal mice; for instance, the 100% mortality in normal and immune mice was induced by treatment with 25 and 100 μg of iron, respectively. This finding suggests that more iron is needed for neutralization of immunity than for satisfaction of the nutritional need of infecting bacteria.

Bacterial fate in sera. In vitro tests of normal and immune sera for antibacterial activity showed that sera had no inhibitory effect and actually supported good growth of virulent SR11 bacteria (Fig. 2). Even sera of mice which were vaccinated with live SL3770 bacteria and then stimulat-

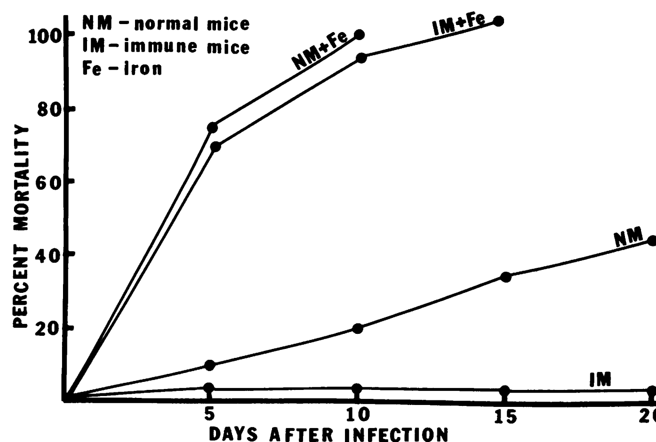

FIG. 1. Percent mortality in iron- or saline-treated normal mice (NM) and mice vaccinated with live SL3770 bacteria (IM) infected with SR11 bacteria (20 mice per group).

ed with killed SR11 bacteria supported good growth of the virulent bacteria. However, normal and immune sera effectively inhibited the growth of avirulent SL3770 bacteria. In agreement with previous results (18), the addition of iron to either normal or immune serum neutralized bacteriostasis and induced uninhibited growth of avirulent bacteria.

In agglutination tests, sera of the SL3770-vaccinated mice did not clump SL3770, SR11, or LT2 bacteria. Sera of SL3770-vaccinated and SR11- or LT2-stimulated mice did not agglutinate SL3770 bacteria but agglutinated virulent bacteria. Sera collected 1 week after the stimulation of mice with killed vaccines weakly agglutinated the homologous bacteria; at 3 weeks, the titer of the agglutinating antibody rose to 100 (data not shown). The addition of various amounts of iron to serological tubes did not change the agglutinating titer.

Iron-resistant immunity. Attempts were made to increase the level of antibacterial immunity in SL3770-vaccinated mice by the elicitation of antibodies to challenge bacteria. Although the stimulation of vaccinated mice with heat-killed virulent bacteria did not increase the level of their resistance to infection significantly, it did induce a state of immunity which was resistant to treatment with iron (Table 1); the stimulation of vaccinated mice with heat-killed avirulent bacteria failed to induce iron-resistant immunity. These results suggest that the activity of iron-resistant immunity relies on the presence of an antibody which can be induced with virulent bacteria. Since the vaccination with heat-killed virulent bacteria by itself induced no protection but elicited formation of some antibodies, the possibility exists that the antibody exerts the antibacterial activity only in the presence of immune factors present in SL3770-vaccinated mice.

Several experiments with SR11- or LT2-stimulated mice showed that each of these virulent strains induces iron-resistant immunity not only against itself but also against the other strain (Table 1). This finding suggests that virulent bacteria have a common antigen which elicits formation of an antibody that plays a protective role in iron-resistant immunity.

The ability of immune sera to impart protection to normal mice was investigated by serum transfer experiments. Results showed that immunity to the challenge with SR11 bacteria was present in recipients of serum collected from SR11- or LT2-stimulated animals (Fig. 3). Mice treated with sera of SL3770-stimulated mice died as rapidly as, or even faster than, mice treated with sera of normal or SL3770-vaccinated mice. Titration of immune sera showed that 0.5 ml of 1:100-diluted serum can protect 50% of iron-treated mice against infection with virulent bacteria. Thus, immune sera, which support unrestricted growth of virulent bacteria in in vitro experiments, protect iron-treated normal mice against the development of lethal infections.

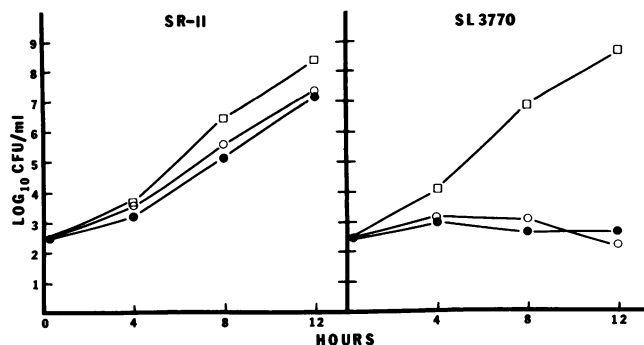

FIG. 2. Growth of *S. typhimurium* SR11 and SL3770 in untreated and iron-enriched sera of normal, immune, and immunologically stimulated mice. Symbols: (○) normal sera; (●) immune or stimulated sera; (□) normal, immune, or stimulated sera with iron.

TABLE 1. Effect of iron on infection with SR11 or LT2 bacteria of mice vaccinated and immunologically stimulated with live or killed vaccines

Bacteria used for:		LD_{50} values for mice treated with[a]:	
Vaccination[b]	Stimulation[c]	Saline	Iron
SL3770		6.1×10^6	$<10^1$
SL3770	SL3770	6.8×10^6	$<10^1$
SL3770	SR11 or LT2	7.5×10^6	7.1×10^6
SR11 or LT2		7.3×10^3	$<10^1$

[a] All mice were injected daily either with saline or with 100 μg of iron. LD_{50}, Mean lethal dose.

[b] Mice were vaccinated twice with 10^6 live SL3770 bacteria or once with 10^8 heat-killed SR11 or LT2 bacteria.

[c] Mice were immunologically stimulated with a sonicated vaccine prepared from 10^8 heat-killed SL3770, SR11, or LT2 bacteria.

The need for specific antibody in iron-resistant immunity was ascertained by two absorption experiments. The protective sera were absorbed twice with washed SR11 bacteria, and each time bacteria were removed by centrifugation and subsequent filtration. The challenge bacteria were added to 0.5 ml of 1:2-diluted absorbed and untreated sera and injected intraperitoneally into iron-treated normal mice. Mice injected with absorbed sera died from the infection within 10 days, whereas mice injected with untreated sera remained alive and well.

Antibody-sensitized bacteria obtained after the first absorption of immune serum with SR11 bacteria were tested for their infectivity in iron-treated normal and vaccinated

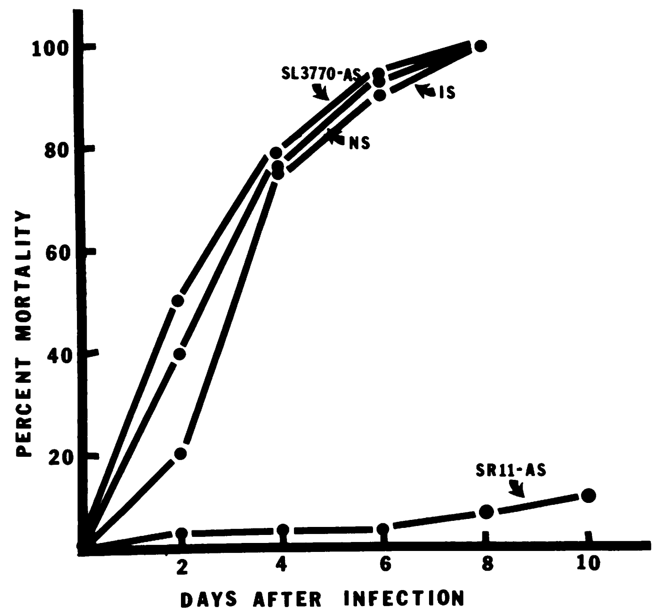

FIG. 3. Percent mortality in iron-treated normal mice infected with 10^3 SR11 bacteria suspended in 0.5 ml of a 1:2 dilution of serum of normal (NS), SL3770-vaccinated (IS), and SL3770-stimulated (SL3770-AS) or SR11-stimulated (SR11-AS) mice. Recipients of serum from LT2-stimulated mice were as resistant as recipients of SR11-AS (20 mice per group).

mice. Results showed that sensitized bacteria were as capable as unsensitized bacteria of producing rapid and lethal infections in iron-treated normal mice (Fig. 4). Only in SL3770-vaccinated, and especially, SL3770-stimulated, mice were the sensitized bacteria partly or totally inhibited; in spite of the treatment with iron, immune serum-sensitized bacteria killed 50% of SL3770-vaccinated and 10% of SL3770-stimulated mice. These results suggest that antibody by itself cannot account for the iron-resistant immunity; to exert its antibacterial activity, it requires the presence of another factor which is present in SL3770-vaccinated mice. Since immune serum cannot inhibit the growth of SR11 bacteria but can transfer the iron-resistant immunity to normal mice, the possibility exists that the immune factor works through antibacterial activity of phagocytic cells.

DISCUSSION

The resistance of vaccinated animals to *S. typhimurium* has been attributed to a nonspecific cellular and a specific humoral immunity. Usually, the cellular immunity was induced by live vaccines prepared from attenuated bacteria; this immunity provided vaccinated animals with solid and durable protection (6). The humoral antibody immunity was induced by killed vaccines prepared from the virulent bacteria; this immunity was highly specific but markedly less effective than the cellular immunity (8). Although killed vaccines induce mainly the humoral immunity, nevertheless they were found to provide a significant protection attributed to either the activity of antibacterial antibody or its synergistic action with activated macrophages (21, 22).

Results presented in this report show that mice vaccinated with live and killed vaccines of *S. typhimurium* develop an immunity which is expressed by the activity of two antibacterial systems. One system is neutralizable by iron and can be induced by the vaccination of animals with live vaccines prepared from avirulent bacteria. The other system is resistant to iron, and it can be induced in animals by a combined effect of live avirulent and killed virulent bacteria. Results suggest that the role of killed vaccine is to elicit the production of specific antibody which can exert its iron-resistant antibacterial activity only in animals sensitized with live bacteria. This essential antibody of iron-resistant immunity can be induced by an antigen common to virulent bacteria and, therefore, it is protective against homologous and heterologous bacteria.

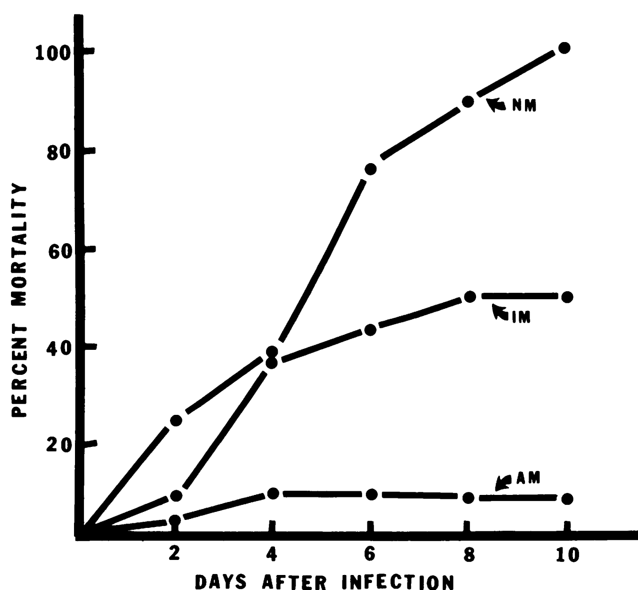

FIG. 4. Percent mortality in iron-treated normal (NM), SL3770-vaccinated (IM), and SL3770-stimulated (AM) mice infected with 10^3 SR11 bacteria sensitized with sera of SR11-activated animals. Normal, vaccinated, and stimulated mice infected with unsensitized bacteria and treated daily with iron died as rapidly as iron-treated normal mice.

The first attempt to neutralize acquired immunity with iron has been made by Sword, who injected mice immune to *Listeria monocytogenes* with iron and observed no neutralization of the immunity (26). Bullen and his associates showed that iron promotes infections with *Pasteurella septica* in mice protected passively with immune serum (4); they suggested that iron neutralizes passively acquired immunity by interfering with antibacterial action of specific antibody, transferrin, and complement which were found to be essential for the immunity (3). The actively acquired immunity has been neutralized in mice vaccinated with *Yersinia pestis* (27) and *Mycobacterium tuberculosis* (13). Results obtained by D. L. Cahall in Youmans' laboratory and by C. A. Golden in our laboratory have shown that iron promotes development of tuberculosis not only in normal but also in immune mice and guinea pigs (14). Subsequently, the work with *S. typhimurium* confirmed the property of iron to neutralize the protective effects of acquired immunity (16, 18).

Present results confirm our previous findings by showing that iron-treated immune mice are killed by virulent bacteria with the same rapidity and frequency as normal mice. Experiments with decreasing numbers of the challenge bacteria showed that the neutralization of antibacterial immunity with iron cannot be attributed to the overwhelming infectious dose. To overcome the immunity with bacteria, it was necessary to challenge immune animals with 10^7 bacteria, whereas lethal infections in iron-treated immune animals were initiated with as few as 10 bacteria. The ability of a few bacteria to kill iron-treated immune animals as rapidly as iron-treated normal animals and much more rapidly than untreated normal animals suggests that iron not only neutralizes the acquired immunity but also promotes bacterial multiplication by its nutritional value. This dual effect of iron was observed clearly in mice injected with decreasing amounts of iron. It has been found that more iron is required to promote bacterial growth in immune than in normal mice; thus, more iron is required for the neutralization of immunity than for the satisfaction of the nutritional need of infecting bacteria.

It has been suggested that immunity to *S. typhimurium* is primarily dependent upon the antibacterial activity of phagocytic cells (6). There is very little information about the effect of iron on intracellular bacteria in immune macrophages or polymorphonuclear cells. It has been shown, however, that in normal macrophages iron promotes unrestricted mycobacterial growth within phagocytic vacuoles (29); in normal phagocytes iron promotes the multiplication of not only facultative intracellular parasites but also obligate extracellular parasites (2). Stimulation of the growth of phagocytized bacteria with iron shows that this essential nutrilite can reach intracellular bacteria, and there it may exert not only a nutritional but also an immunity-neutralizing effect (12). Although iron does not interfere with the development of delayed hypersensitivity or its elicitation in sensitive guinea pigs (16), preliminary results with infected tissue

cultures of normal and immune macrophages suggested that iron interferes with the antibacterial activity of immune cells (S. K. Wagner, M.S. thesis, Miami University, Oxford, Ohio, 1983). The ability of iron to interfere with the activity of antibacterial factors against facultative intracellular parasites but not with delayed hypersensitivity supports the concept of the separate nature of cellular immunity and delayed hypersensitivity (31).

Investigation of the effect of iron on humoral immunity has shown that iron does not interfere with either the production or the bactericidal activity of antibodies (5, 24); also, iron does not interfere with the production or activity of hemolysin and complement (16). In terms of antibody-producing cells, these results indicate that iron exerts no suppressive activity on the B-lymphocyte–plasma cell system and T-helper cells.

The inability of iron to interfere with lytic activity of the antibody-complement system does not eliminate a possibility that iron may interfere with some other protective activity of antibody. It has been shown that an antibody to iron-binding enterobactin interferes with the iron-recruiting activity of this bacterial chelator (20; M. W. Mellencamp, J. Wasynczuk, J. T. Kvach, and I. Kochan Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B84 p. 31) or with its synthesis (25). In the presence of transferrin, antibodies to the bacterial monosaccharide colitose were found to be able to inhibit bacterial growth by deprivation of iron (7). In spite of these bacterial growth-inhibiting effects of antibodies, virulent bacteria were found to be able to multiply in sera collected from immune animals (16).

Results presented in this report show that virulent SR11 bacteria can multiply at the same rate in sera collected from normal, immune, and immunologically stimulated mice. Although inactive in test tubes, serum of SR11- or LT2-stimulated mice protected iron-treated normal mice against the challenge with virulent bacteria. The absorption experiments showed that iron-resistant immunity is dependent upon the activity of antibacterial antibody induced in mice by the vaccination with heat-killed virulent bacteria. The multiplication of antibody-sensitized SR11 bacteria in iron-treated normal animals revealed that the antibody by itself cannot account for the iron-resistant immunity. Such sensitized bacteria were, however, inhibited in SL3770-vaccinated and, especially, SL3770-stimulated mice which, when infected with unsensitized bacteria, were as susceptible to the infection-inducing activity of iron as normal mice.

It seems that the protective serum of immunologically stimulated mice elicits antibacterial reactions in normal mice by factors which by themselves are unable to exert antibacterial activity. It is quite likely that such passively acquired immunity is expressed by the antibacterial activity of phagocytic cells stimulated by lymphokines and antibodies of immune serum.

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