# Antimicrobial Defense Mechanisms in the *Salmonella typhimurium* Associated Ex-germfree Rat[1] (34780)

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In recent years the gnotobiotic system has been used extensively to study the various physiological, immunological, and biochemical aspects of antimicrobial defense. The germfree animal, when maintained on a diet relatively free of antigenic materials, is especially suited for studying the activation of defense mechanisms. As one rather extreme approach, the association of germfree rats with *Salmonella typhimurium* was chosen since this organism constitutes a severe challenge to the ex-germfree host and may lead to 25% death during the first 2 weeks of association.

Earlier reports had indicated a relatively slow change in the rat serum globulin pattern, especially in the γ-globulin fraction, following association with a "normal" flora or its components (1, 2). Specific agglutinins were detectable only after 2 weeks of association (3). The present study gives immunological and serological data covering the first 25 days of monoassociation with *S. typhimurium ND 750A*. The data again suggest that humoral immune mechanisms in the rat, even under severe pathogenic stress, are slow to be activated. Acute changes were found in albumin, α- and β-globulin concentrations, and in the concentration of a presumably nonimmune γ-globulin of low electrophoretic mobility. An early and pronounced increase in phagocytic ability indicated a primary role of phagocytosis in the antimicrobial defense of the rat.

*Methods.* Female rats from the Lobund closely bred germfree colony (Wistar origin) were used. In earlier series the rats were fed steam sterilized practical type diet L 462 (4), in later series commercial rat diet 5010 C (Ralston Purina Co., St. Louis, Mo.). The animals were housed in Trexler type plastic isolators. At the age of approximately 90 days they were monoassociated with *Salmonella typhimurium ND 750A* by oral inoculation from an 18-hr culture, while the remainder of the culture was sprinkled on the diet. At specified times the animals were killed by exsanguination from the heart under pentobarbital anesthesia, and organs were removed and weighed. The data on conventional rats was obtained from genetically closely related animals maintained on the same sterilized diets in the open animal house.

Cellulose acetate electrophoresis was performed on 3 × 15-cm strips using a sodium barbital buffer, pH 8.6 (ionic strength 0.06). The strips were stained with amido black, and after destaining, were read with a Chromoscan reflectance densitometer (Joyce, Loebl and Co., Burlington, Massachusetts). Total protein concentration was determined with a serum protein refractometer. Immunoelectrophoresis was performed on 2.5 × 7.5-cm glass slides. Sodium barbital buffer, pH 8.2 (ionic strength 0.1), was used, with Ionagar No. 2 (Consolidated Laboratories, Inc., Chicago Heights, Illinois) made up to 0.75% in the buffer as a supporting medium.

Agglutination titers against the homologous organism were determined using routine procedures. They were expressed on the usual log 2 scale. The curve thus obtained was subsequently transformed to fit a linear dilution scale in order to express the linear relationship between association time and titer.

Carbon clearance was determined with the technique originally described by Biozzi and co-workers (5). Blood samples were taken

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from the ophthalmic venus plexus. Results are expressed as the phagocytic index $K$ (slope of the plot of log concentration versus time).

*Results.* Within 24 hr after monoassociation the bacterial count reached approximately $10^9$/g of cecal content. Thereafter the microbial population remained stable, not only during the 4-week experimental period described here, but also in other experiments covering periods up to 3 months. During the second, third, and sometimes fourth day it was possible to culture viable salmonella from liver and spleen. Thereafter no sign of bacterial penetration could be found.

Figure 1 indicates the morphological reaction of the reticuloendothelial system in rats maintained on diet L 462. Rats reared on diet 5010 C showed comparable results in this and all other aspects reported in this paper. The increase in weight of the spleen and lymph nodes started within 24 hr after monoassociation and reaches its maximum after approximately 10 days. Thereafter the weights declined to almost preassociation values at the end of the experimental period. Throughout this period, the dry weight of the spleen remained constant; the daily means averaged to 22.2 ± 0.6% (SD).

Throughout the experimental period the total serum protein content remained essentially constant and comparable to the level found in the conventional rat. An exception was found at day 2 when the average concentration of 5.4% total protein (4 animals) was

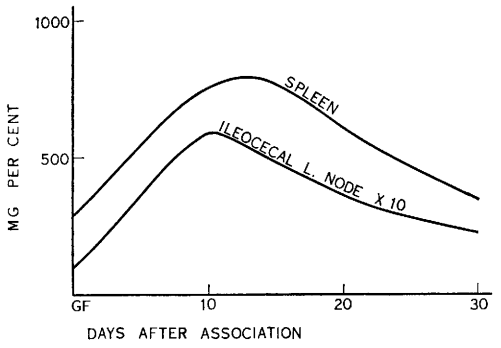


FIG. 1. Weight of spleen and lymph nodes. Germfree rats monoassociated at day 0 with viable culture of *Salmonella typhimurium* ND 750A.

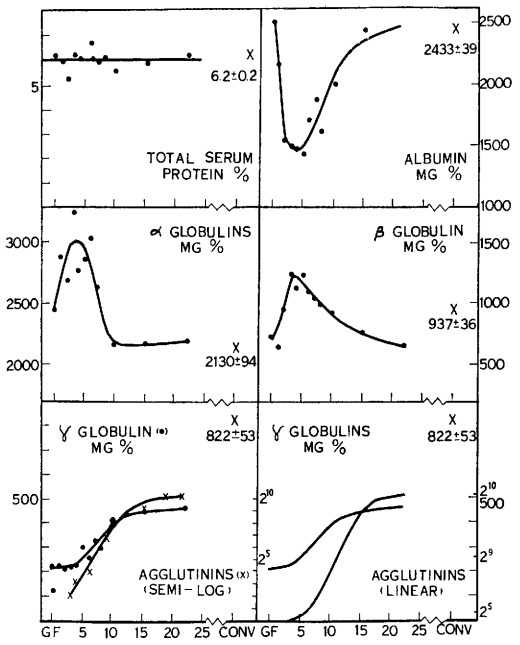


FIG. 2. Serum protein and agglutinin concentrations after monoassociation with *S. typhimurium*.

significantly below the average value found for comparable conventional animals (Fig. 2). Conventional young adult rats maintained on diet L 462 showed an average concentration and standard error of 6.2 ± 0.2%.

The serum albumin concentration decreased within 24 hr after monoassociation and reached its minimum (56% of preassociation value) at day 4. $\alpha$- and $\beta$-globulin concentrations reached maxima (121 and 170% of preassociation value) in the same 4-day period (Fig. 2). Two weeks after monoassociation, albumin, $\alpha$- and $\beta$-globulins concentrations had returned to preassociation levels. The concentration of the $\gamma$-globulin fraction as defined by the present technique started to increase around the fifth day and increased from preassociation levels of approximately 200 mg/100 ml to double that concentration between days 5 and 10. Thereafter the increase was slow and suggested a plateau of approximately 500 mg/100 ml. This value was substantially lower than the average of 822 ± 53 mg/100 ml (SE) found in conventional rats. Because immunoelectrophoresis has indicated that the gamma globulin fraction as determined by cellulose acetate elec-

trophoresis contains only part of IgG, with $IgG_1$, IgM and IgA contributing mainly to the $\beta$-globulin range (6, 7), the aforementioned gamma globulin concentrations are proportionate but not absolute values.

Specific agglutinins appeared at the third day of association. Since agglutinating activity involves both quantity and quality of the antibody, the linear as well as the usual semilogarithmic relationship with time have been indicated. The linear data plot suggests that the major increase in agglutinating activity of the serum occurs parallel with the increase in gamma globulin.

An analysis of the changes in the immunoelectrophoresis pattern following association with *S. typhimurium* is given in Fig. 3. The site of application and the transferrin arc have been indicated for reference. All lines entering the space under the transferrin arc from the $\alpha$-globulin range, including the $\beta_{1C}$ line, have been omitted. In conventional rat serum four "gamma" fractions were usually indicated besides the IgG line, designated here as $\gamma_a$, $\gamma_b$ $\gamma_c$, and $\gamma_x$. Upon fractionation of whole serum with Sephadex 200, fraction $\gamma_a$ was identified by immunoelectrophoresis in the first, macroglobular peak of the elution diagram and apparently consists of IgM. This fraction was not always visible in the original serum pattern. Fraction $\gamma_b$ appeared, together with IgG, in the second major peak, while $\gamma_c$ has been tentatively identified in the third containing albumin and transferrin as major fractions. So far the position of $\gamma_x$ in the Sephadex-200 elution diagram has not been established. The pattern of the germfree rat maintained on these practical type diets showed essentially the same protein fractions in the $\beta$–$\gamma$ range as the conventional rat, although intensity and position of the arcs indicated lower concentrations. A possible exception was the arc in the slower $\gamma$ range ($\gamma_x$). This arc, which in germfree rat serum often appears totally detached from the IgG line, seems to occur in the same low order of concentration in the blood of both germfree and conventional rats.

Upon monoassociation the first major change was seen in the $\gamma_x$ fraction. The intensity of the arc reached a maximum approximately 4 days later, then decreased to its preassociation appearance at approximately day 10. Arcs $\gamma_b$ and $\gamma_c$ both indicated moderate increases in size and intensity within 2 days of monoassociation. Arc $\gamma_a$ was not always sharply indicated, but became quite pronounced at day 6 when it attached itself to a new arc which had appeared underneath the now fading "original" IgG line, and the still heavier than normal $\gamma_x$ arc. Spur formation suggested only partial identity between fraction $\gamma_a$ and the fraction indicated by the newly emerged arc, with additional antigenic determinants apparent only in the latter fraction. Subsequently this combination of lines

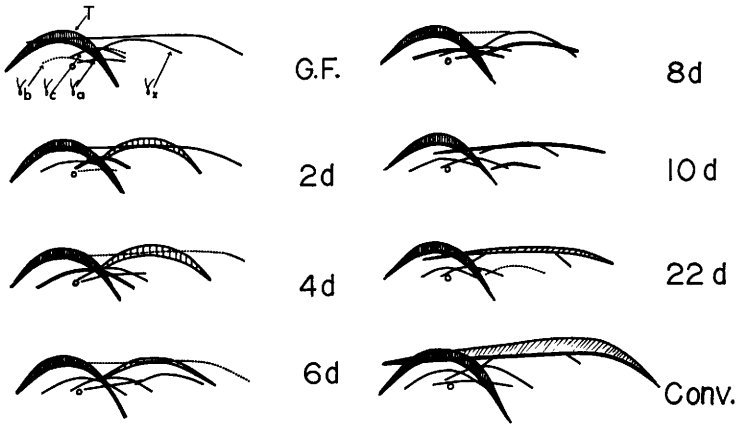


FIG. 3. Immunoelectrophoretic pattern of gamma globulin range of rat serum after association with *S. typhimurium*. Transferrin arc (T) and site of application indicated. For $\gamma_a$, $\gamma_b$, $\gamma_c$, and $\gamma_x$ see text.

increased in intensity, and appeared to form a continuum. At day 10 the "original" IgG line had disappeared, but the new combination showed as one strong line covering almost the range of the original IgG. At the same time another arc appeared in a position in the slower $\gamma$ range almost identical to that of the arc appearing at day 6. This arc could be identified in patterns at day 10, 15, and 22, but was not found in the patterns of either germfree or conventional rats.

Carbon clearance data are given in Fig. 4. *K* values found for germfree rats are only slightly lower than those shown by comparable conventional rats. Upon association a rapid rise in *K* value occurs, which at its maximum represents an approximately 100-fold increase over the value found for the germfree animal. After the first week of association *K* values were found to decline rapidly.

Between 3 and 4 weeks after monoassociation the rats regained all outward appearances of healthy animals. Although the intestinal *S. typhimurium* population remained at the level established early in the experiment, the animals regained most of their preassociation characteristics.

*Discussion.* In general the germfree rat withstands microbial association well. Brought into a conventional environment, or associated with specific microbial species, the animal usually demonstrates no more than passing discomfort characterized by diarrhea and some transitory loss of weight (1). Monoassociation with *Staphylococcus albus*, *Clostridium perfringens*, *Streptococcus faecalis*, or *Lactobacillus casei* (2, 3) produces no demonstrable increase in $\gamma$-globulin, and homologous agglutinins appear relatively late (2). Association with *S. typhimurium* (ND No. 750A), however, constitutes an unusually severe challenge, as indicated by loss of weight and sometimes death of the animals. Formation of agglutinating antibody is paralleled by a substantial increase in serum $\gamma$-globulin, although the response is a relatively slow one. Germfree mice stimulated with formalinized *S. typhimurium* reached maximum agglutinin titers at the end of the first week (8). In the present study germfree rats monoassociated with the same organism needed 2–3 weeks to reach maximum titers of similar magnitude. Earlier findings had indicated no obvious relation between the time of appearance of specific agglutinin titers and the increase in gamma globulin levels after monoassociation (3).

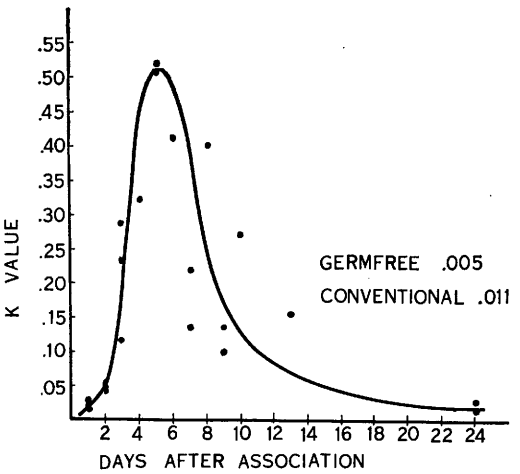


FIG. 4. Carbon clearance in ex-germfree rats monoassociated with *S. typhimurium*. Average phagocytic index (*K* value) for germfree and conventional rats given for comparison.

Immunoelectrophoresis of germfree rat serum shows a light but clearly indicated IgG line (Fig. 3). During the first week of monoassociation, this line fades, and is eventually replaced by a line which appears to develop from an arc originating in the slow $\gamma$ range by the end of the first week. Development and intensity of the "new" arc suggest a continuous increase in concentration of this fraction which, at least at one time (day 6), shows partial identity with fraction $\gamma_a$, tentatively identified as IgM. The event coincides with a major and permanent increase in electrophoretically determined serum $\gamma$-globulin that starts at the end of the first week, and would seem related to the increase in specific agglutination titer (Fig. 3). The transitory appearance of another arc in approximately the same position during the second and third week of association remains unexplained.

Factors other than specific humoral antibody appear to play an important part in the

early adaptation phases of the ex-germfree rat. Spleen and lymph nodes demonstrate almost instantaneous hyperplasia. Serum $\alpha$- and $\beta$-globulins show rapid increases in concentration and early maximum values. These have returned to preassociation levels at the time when the $\gamma$-globulin concentration has reached its new plateau of approximately 500 mg/100 ml, and homologous agglutinin titers have attained their maximum value. Increased production of $\alpha_2$-macroglobulin (9), and of haptoglobulin (7, 10) and transferrin (10) under the influence of bacterial endotoxins has been reported and would explain the transient increase in $\alpha$- and $\beta$-globulins. However, the drastic fall in serum albumin, presumably at least in part under direct influence of the *S. typhimurium* exdotoxin (11), would require a certain compensatory globulin production to maintain the colloid-osmotic value of the blood. Thus part of the increase in $\alpha$- and/or $\beta$-globulin levels might be regarded as the result of nonspecific feedback.

Immunoelectrophoresis indicates that the $\gamma_x$ concentration follows a pattern of rapid rise and decline similar to that of $\alpha$- and $\beta$-globulin, although this observation is by necessity only semiquantitative. This would classify this slow moving gamma globulin fraction as an acute phase protein rather than as an immune protein. Electrophoretic mobility and behavior are suggestive of a protein with CRP characteristics. CRP and $C_x$RP, which electrophoretically move in the $\gamma$-globulin range (10), have been classified as acute phase proteins (12, 13). Williams and Wemyss (7), and also Fahey and co-worker (14), have decribed a $\gamma_x$ fraction of supposedly nonimmune character in the slow $\gamma$ range in mice. Similar protein fractions have been described in the guinea pig and the piglet (15, 16).

Nonspecific defense mechanisms, seemingly correlated with the occurrence of acute phase proteins in the $\alpha$- and $\gamma$-globulin fractions, appear to bear the brunt of the early microbial impact on the ex-germfree rat. The bacteremia that occurred upon association never extended beyond the fourth day. The carbon clearance data, which in germfree rats showed $K$ values of 0.005 and which rose to a maximum value of approximately 0.50 6 days after association (Fig. 4) suggest that increased phagocytic ability constitutes an important factor in early antimicrobial defense. Phagocytosis uptake is possibly enhanced by the observed increase in $\alpha$-globulin (17).

Aided by nonhumoral as well as by humoral defense mechanisms, the ex-germfree rat adjusts well to association with *S. typhimurium*. While nonspecific mechanisms aid to control the first impact of the association, specifically directed circulating antibody appears to play a major role in maintaining the ecological equilibrium between host and associate. Between 3 and 4 weeks after monoassociation the ex-germfree rat regains most of its preassociation characteristics. Mesenteric (Fig. 1) and submandibular lymph nodes are only slightly larger than in the germfree animal, and definitely smaller than in conventional controls. $\alpha$- and $\beta$-globulin levels, and the appearance of the $\gamma_x$ arc are comparable to those in the germfree rat. Only the increased $\gamma$-globulin level and the persistence of homologous agglutinins point to the continuing presence of *S. typhimurium*. However, Wiseman and Gordon (18) report that microbial samples cultured from these animals have lost none of their pathogenic properties upon reintroduction into germfree rats.

*Summary.* Germfree rats were monoassociated with *Salmonella typhimurium*. Early responses included increases in serum $\alpha$- and $\beta$-globulins and a decrease in albumin. A presumably nonimmune $\gamma$-globulin fraction of slow electrophoretic mobility showed a similar increase, while carbon clearance values increased 50- to 100-fold. During the second week of association $\gamma$-globulin levels increased substantially. This was paralleled by a considerable increase in homologous agglutinating antibody, which first became evident 3 days after association. Both gamma globulin concentration and agglutinating antibody reached a plateau after the second week. At the end of that week, albumin, $\alpha$- and $\beta$-globulin levels, and the carbon clearance index had returned to practically preassociation

levels. The nonimmune gamma globulin fraction likewise had regained its preassociation immunoelectrophoretic appearance. It is concluded that while nonspecific defense mechanisms are a major protective factor during the first week after association of the ex-germfree rat, specific antibody production protects the animal during and especially after the second week and enables it to adjust fully to the association with a strongly pathogenic microorganism.

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