# GAMMA GLOBULIN PRODUCTION IN GERMFREE RATS AFTER BACTERIAL CONTAMINATION*

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In an earlier communication (1) it was demonstrated that the electrophoretic gamma globulin fraction was markedly decreased and the beta fraction slightly decreased in germfree rats. Wostmann (2) found hypogammaglobulinemia in germfree rats, Swiss mice, and guinea-pigs. Thorbecke *et al.* (3) had earlier shown that the gamma globulin values in germfree chickens remain low as the animals mature, while normal stock chickens show a steady increase with time until about 30 per cent of the total serum protein is taken up by the gamma globulin.

It is impossible to decide from these experiments whether stimulation by exogenous antigens is necessary for the formation of all gamma globulins or only for the bulk of the gamma globulin since no animals have as yet been raised on a diet reasonably free from antigenic substances.

In this paper the changes in the electrophoretic and the immunologic gamma globulin fractions obtained in germfree rats after exposure to their normal microbial flora will be reported.

## *Materials and Methods*

The germfree rats were reared according to the technique described elsewhere (4, 5).

*Series A* comprised 4 animals (litter 1) with a mean weight of 207 gm. at an age of 91 days. During care of the animals one glove of the germfree apparatus ruptured. After 2 days it was proven that the cage had been infected with *Bacillus subtilis*; thus the animals were removed on the 3rd day and transferred to the animal room for ordinary rats. Day 0 represents the day when the glove ruptured. Twelve blood samples were collected from each animal during a follow-up period of 18 weeks.

*Series B* comprised 28 animals in 6 age groups (litters 2 to 7). The material is presented in Table I and Fig. 2. The experiment was started when a germfree apparatus containing all these animals was infected with a mold. This infection was traced to a new, badly functioning food autoclave and therefore the time of the mold infection could be determined with certainty. When the animals had been exposed to the mold for 6 days, they were transferred to the room for breeding of normal rats and were thereby exposed to the normal microbial flora.

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Day 0 on the diagram represents the day when the animals were infected with mold and day 7 was the first day of exposure to the full flora. Blood samples were taken weekly from 10 animals. Blood was sampled less regularly from the remaining 18 animals. The follow-up period after infection was 8 weeks. Some rats were killed during the experimental period for morphological studies.

*Series C* comprised 3 germfree rats (litter 8) with a mean weight of 240 gm. at an age of 106 days. They were followed for a 9 week period after exposure to *Staphylococcus albus.*

*Series D* comprised 3 litters of newborn ordinary rats.

*Electrophoretic analysis* was performed as described earlier (1). Some serum samples were contaminated with hemoglobin which interfered with the electrophoretic analysis of the alpha-2 and beta fractions. Others were slightly lipemic, which did not seem to influence the results.

TABLE I

*Data Concerning Animals in Series B*

Age and weight figures refer to day of infection. Figures within ( ) are approximate.

| Litter No. | Age | Germfree generation | Mean weight | No. of animals | No. of animals with weekly blood samples | No. of blood samples |
|---|---|---|---|---|---|---|
| | *days* | | *gm.* | | | |
| 2 | 331 | 5 | 278 | 2 | 2 | 16 |
| 3 | 158 | 6 | 271 | 3 | 1 | 17 |
| 4 | 55 | 7 | 122 | 5 | 4 | 34 |
| 5 | 43 | 6 | 74 | 7 | 3 | 40 |
| 6 | 35 | 6 | (70) | 4 | 0 | 28 |
| 7 | 13 | 7 | (26) | 7 | 0 | 43 |
| | | | | 28 | 10 | 178 |

The sera were kept at about −15°C. until analysis, which was performed in series to minimize the systematic analytical errors.

*Isolation of Rat Gamma Globulin.*—Gamma globulin was prepared from pooled normal rat sera according to the technique of Hořejši and Smetana (6). Three parts of rivanol solution (0.4 per cent adjusted to pH 9.0) was added with stirring to one volume of serum. The precipitate was centrifuged off. The supernatant was treated with activated carbon until the rivanol colour was eliminated. Solid ammonium sulfate was added to the clear solution to obtain a final saturation of 40 per cent. The precipitate was spun down and resuspended in ammonium sulfate of 40 per cent saturation and recentrifuged. The precipitate was dissolved in water after careful draining of the tubes and dialyzed against normal saline.

When paper electrophoresis was run on a concentrated solution (about 5 per cent) only gamma globulins were found.

*Chicken Anti-Rat Gamma Globulin Serum.*—The gamma globulin preparation was used for immunization of chickens according to Goodman *et al.* (7). 40 mg. gamma globulin per kg. body weight was injected intravenously. The animals were extravasated by infusion of about 50 ml. physiological saline 1 week later. The serum was diluted with equal parts of 16 per cent NaCl. Sera from 3 chickens were pooled. The same batch was used for all experiments. The antiserum was distributed in ampoules and kept at −15°C. until used.

*Determination of Immunologic Gamma Globulins with Quantitative Precipitation.*—One of the techniques of Goodman *et al.* (7) was mainly followed. 0.2 ml. gamma globulin or serum dilution in 8 per cent NaCl was mixed with 0.4 ml. antiserum. The tubes were placed 1 hour

at 37°C. followed by ½ hour at +4°C. and then centrifuged at about 20,000 *g* at +4°C. The supernatant was decanted. The precipitate was suspended in 3 ml. of 8 per cent NaCl at +4°C. and recentrifuged. This washing step was repeated once and the tubes were allowed to drain upside down on a filter paper. The remaining fluid at the collar of the tubes was eliminated with a dry filter paper. 0.8 ml. 0.1 N NaOH was used to dissolve the precipitate and the extinction was measured at 280 mμ 10 to 15 minutes later in Beckman DU 1 cm. cuvettes supplied with an adapter for small volumes.

A standard curve was constructed using rat gamma globulin diluted in 8 per cent NaCl (Fig. 1). When working with a gamma globulin dilution of less than 15 mg. per 100 ml., antibody excess was certain with this antiserum and the absorption values were of a suitable magnitude.

The rat sera were diluted with 8 per cent NaCl to obtain a gamma concentration within the range of 2 to 15 mg. per 100 ml. A quantity of 0.01 ml. rat serum was diluted as routine

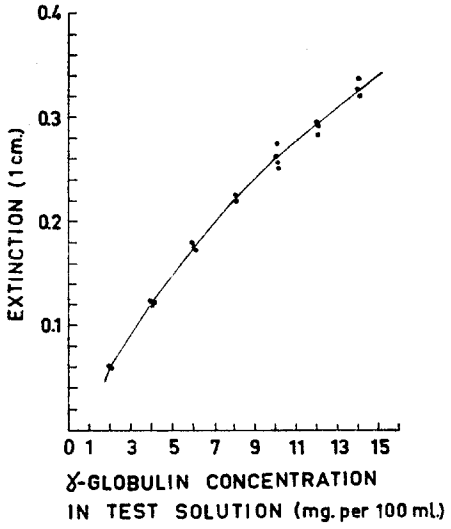


FIG. 1. Calibration curve for gamma globulin determination with our chicken anti-rat gamma globulin serum and purified rat gamma globulin. The points plotted derive from 4 different experiments.

with 1 ml. of 8 per cent NaCl. Gamma globulin standard solutions were included in each series. On repeated analysis values outside ±5 per cent were seldom obtained. At normal rat gamma globulin concentration the reproducibility is thus better than on electrophoretic analysis.

## RESULTS

No highly significant changes were observed in the electrophoretic serum albumin, alpha-1, alpha-2, or beta globulin values comparing the early and late non-germfree period (Table II). The subnormal alpha-2 level in germfree rats and the normal level observed by Wostmann (2) after contamination of the animals with a "normal" cecal flora were not confirmed.

In the two series studied all the animals had pronounced hypogammaglobulinemia when they were exposed to the normal microbial flora. The gamma

TABLE II

*Comparison between Serum Protein and Electrophoretic Values in the Early and Late Non-Germfree Period of Rats Belonging to Series B*

1–3 designates sera from rats non-germfree for 0 to 2 weeks and 6–8 designates 5 to 7 weeks.

| | Total serum protein | | Albumin | | Alpha-1 globulin | | Alpha-2 globulin | | Beta globulin | |
|---|---|---|---|---|---|---|---|---|---|---|
| Non-germ-free period | 1–3 | 6–8 | 1–3 | 6–8 | 1–3 | 6–8 | 1–3 | 6–8 | 1–3 | 6–8 |
| Mean value | 6.2 | 6.4 | 3.7 | 3.5 | 0.93 | 0.86 | 0.73 | 0.72 | 0.77 | 0.81 |
| Standard errors of the mean | 0.055 | 0.077 | 0.057 | 0.047 | 0.019 | 0.022 | 0.019 | 0.016 | 0.018 | 0.016 |
| S.D. | 0.44 | 0.58 | 0.45 | 0.37 | 0.15 | 0.18 | 0.15 | 0.13 | 0.14 | 0.13 |

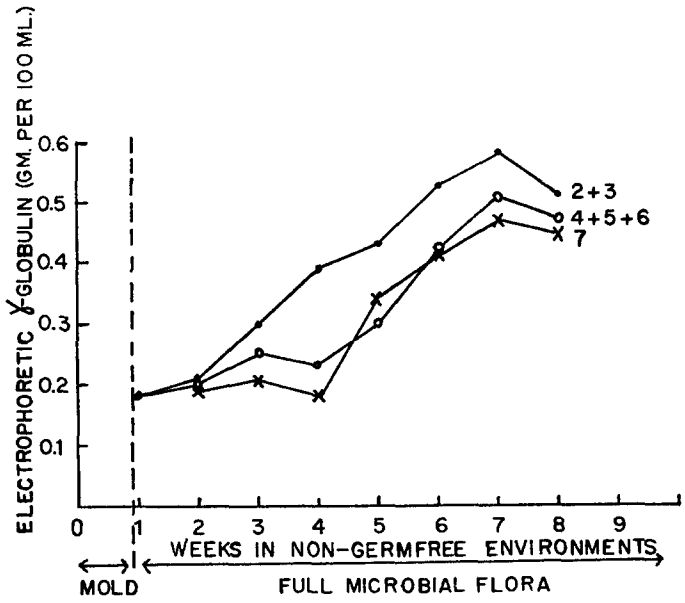


FIG. 2. Changes in gamma globulin concentration after exposure of three weight classes of germfree rats (series B) first to mold (6 days) and then to full normal microbial flora. The numbers to the right refer to the litter number.

globulin level became normal in all animals during the experimental period. The reaction pattern varied with the body weight. The results are presented in Figs. 2 and 3 in which the mean values of different groups are plotted.

Animals of litters 2 and 3 (series B) were full-grown and had the same mean weight in spite of age differences. Their reaction pattern was identical which is why they are presented as one group. Litters 4, 5, and 6 of young rats have also been treated as a single population since no difference was noticeable. Litter 7 comprises the youngest group which is why the results are presented separately. It is apparent from the figures that animals 6 months to 1 year of age (compare Table I) showed a more continous increase of the gamma globulin level after exposure to the normal flora than did the younger, growing animals

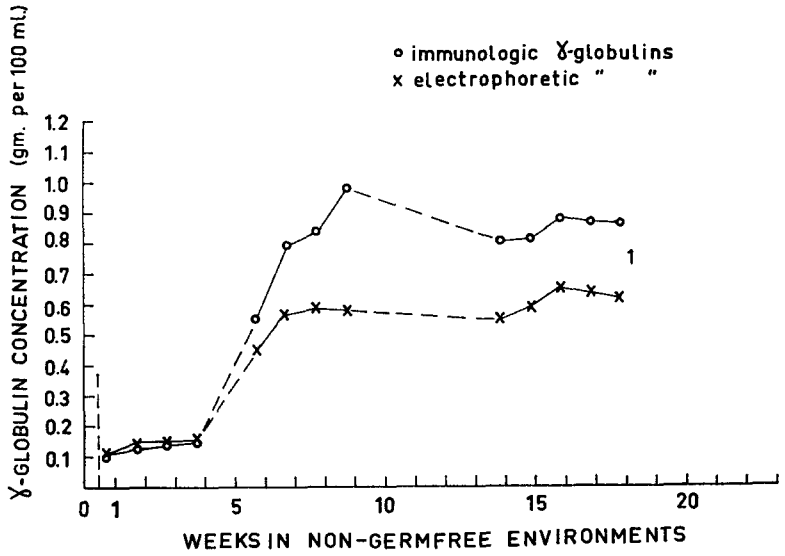


FIG. 3. Changes in electrophoretic and immunologic gamma globulin concentration after exposure of germfree rats (series A, litter 1) first to *Bacillus subtilis* 3 days and then to normal microbial flora.

studied (initial age 2 weeks to 3 months). These rapidly growing animals showed a very slow, although statistically not significant, gamma globulin increase during the first 4 weeks. During the following 3 week period they became normal.

All serum samples from the animals in series A (litter 1) were also analyzed for immunologic gamma globulins. The mean values are presented in Fig. 3 which for comparison also contains the electrophoretic gamma globulins. Immunologic gamma globulin analysis was not performed on the numerous sera derived from rats in series B. Before it was apparent that the full-grown and growing rats did not react identically, equal quantities of sera from all rats (series B) exposed to the normal flora for the same period were pooled for immunologic gamma globulin analysis. The results are given in Fig. 4. The

normal immunologic gamma globulin in rats was found to be about 0.8 to 1.0 gm. per 100 ml. against 0.5 to 0.6 gm. per 100 ml. for the electrophoretic gamma

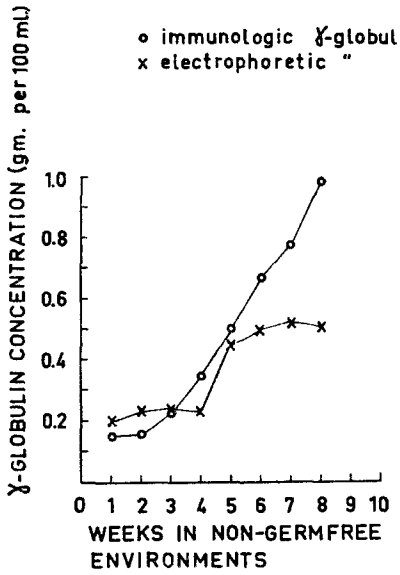


FIG. 4. Comparison between immunologic and electrophoretic gamma globulin values in pooled sera from all animals belonging to series B.

TABLE III

*Electrophoretic Gamma Globulin Values before and after Exposure of 3 Germfree Rats (Series C) to Staphylococcus albus*

| Day | Litter 8 | | |
|---|---|---|---|
| | No. 1 | No. 2 | No. 3 |
| | *gm./100 ml.* | *gm./100 ml.* | *gm./100 ml.* |
| 0 | 0.23 | 0.29 | 0.18 |
| 37 | 0.24 | 0.20 | 0.20 |
| 46 | 0.29 | 0.25 | 0.20 |
| 53 | 0.27 | 0.19 | 0.26 |
| 60 | 0.25 | 0.25 | 0.26 |
| 67 | 0.21 | 0.24 | 0.24 |

globulins. Sera from germfree animals (Figs. 3 and 4) showed only 0.1 to 0.15 gm. immunologic gamma globulin per 100 ml., thus comparatively less immunologic than electrophoretic gamma globulin. The increase of both types followed the same time course during the first weeks but in both series the

increase of immunologic gamma globulins went on for a somewhat longer period than that of the electrophoretic.

Series C (litter 8) comprised 3 germfree rats followed 10 weeks after exposure to *Staphylococcus albus*. No significant change was recorded in the electrophoretic gamma globulin fractions (Table III).

Sera from three litters of very young ordinary rats were also analyzed electrophoretically to follow the gamma globulin variation in lactating and weanling rats under normal environmental conditions. Repeated blood sampling from each animal was technically almost impossible. Thus the young rats in each litter had to be killed at 1 week intervals. The result is given in Fig. 4 which for comparison also contains a curve calculated on basis of gain of weight showing the expected gamma globulin decline if no gamma globulins are formed or absorbed after the first postnatal day. No statistically significant change in the gamma globulin level was observed during the first 7 weeks. The material is, however, too small for definite conclusions since rather great individual variations were observed.

## DISCUSSION

The sera from the germfree rats in these series had a clear cut subnormal electrophoretic gamma globulin concentration as observed earlier. With immunologic technique a more pronounced hypogammaglobulinemia was observed than with the paper electrophoretic technique. All fractions moved towards the anode with electrophoretic technique used. Trailing therefore results in erroneously high electrophoretic values, which explains the discrepancy observed.

On immunoelectrophoresis of rat sera with Scheidegger's technique (8) using chicken anti-rat gamma globulin serum a single precipitation line from slow gamma fading out into alpha-1 was obtained, thus demonstrating a somewhat wider distribution of immunologic gamma globulin than found in human sera (9, 10). In normal human sera (10) the immunologic gamma globulin concentration is about 25 per cent higher than the electrophoretic but in rat sera it is about 50 per cent higher.

When growing germfree rats are exposed to the full normal flora the gamma globulin level increases very slowly during the first 3 to 4 weeks. The small, slow increase during this period is significant when the immunologic technique of analysis is used, showing that the increase probably does not depend on a change in water distribution since no change is found in the albumin fraction. After this period the level increases rapidly and reaches the normal electrophoretic gamma globulin level during the next 3 to 4 weeks. In full-grown rats a less pronounced lag phase was observed before the more rapid phase of normalization occurred. The biological reason for the difference between growing and adult rats has not been elucidated. When one group of germfree rats was contaminated with *Staphylococcus albus*, no change was observed in the elec-

trophoretic gamma globulin concentration, indicating that not all bacterial cells when reaching the animals on natural routes are equivalent as stimulators for gamma globulin production.

The relative lag phase in appearance of gamma globulins after exposure of the animals to the normal flora contrasts to the rapid appearance of antibodies occurring when normal rats are immunized by parental administration of known antigens. Antibodies against sheep red cells or typhoid H-antigen appear in rats after about 2 days and optimal concentrations are obtained after 5 to 7 days (11, 12). Depending on the antigen used, the antibodies may remain

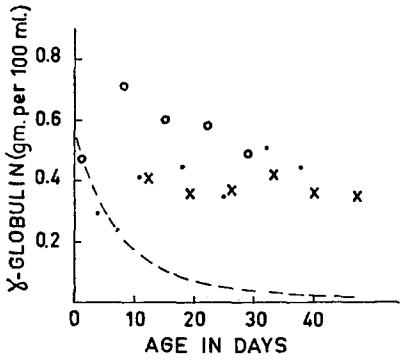


FIG. 5. Electrophoretic gamma globulin values during the postnatal period in rats. Identical signs have been used for young rats belonging to the same litter. The broken line represents the expected gamma globulin decline calculated on gain of weight if it is assumed that no gamma globulins are formed or absorbed after the first postnatal days.

on a high level for a long period or for only a few days. It should be pointed out that the germfree animals regularly develop slight acute gastroenteritis during the first days after contact with the normal microbial flora but not to the same degree after contact with mold, *Bacillus subtilis* or *Staphylococcus albus*. The time difference in antibody formation in normal rats and gamma globulin formation in ex-germfree rats may partly be explained by the route of antigen administration and by differences in quantities of high potent antigens entering the body. Further experiments are necessary before definite conclusions can be drawn.

In newborn human babies it is known that the gamma globulin production is very low during the first months of life and then speeds up. This is a phenomenon similar to that found in ex-germfree rats. We therefore studied the gamma globulin concentration of serum in a series of newborn and young rats, but from these results there seems to be no pronounced lag phase before gamma globulin production starts after birth (Fig. 5). The gamma globulin level of the young rats remains on an approximate steady-state in spite of a

very rapid growth indicating either high activity of the plasma cell system or possibly gamma globulin absorption from the mothers' milk for a longer period than has been observed in other species such as pig, cow, and horse (13).

The functional capacity per body weight of the plasma cell system in newborn rats is thus probably much higher than in the ex-germfree rats during the initial period. Our results may support the hypothesis that the germfree animals have few cells ready to form gamma globulins on stimulation. They have clear cut subnormal amounts of plasma cells in their lymphoid apparatus. The normalization of the gamma globulin level is preceded by a proliferation of the different types of plasma cells. (Details of the histological studies will be presented in a forthcoming paper). It may be assumed that a more or less continuous stimulation of the plasma cell system by antigens derived from the normal flora influences the proliferation rate of these cells. Penetration of less antigens into the body may secondarily result in decreasing cell mass in the plasma cell system and therefore in a decreased functional capacity with regard to gamma globulin and antibody formation.

The results of electrophoretic and immunologic gamma globulin analysis have principally given the same results but the normalization of the immunologic gamma globulin fraction seems to occur somewhat more slowly than the normalization of the electrophoretic gamma globulins.

## SUMMARY

The earlier observed pronounced hypogammaglobulinemia in germfree rats of different ages has been confirmed. Using an immunologic technique the concentration of immunologic gamma globulins were found to vary between 10 and 15 per cent of the values observed in ordinary rats. Upon contamination of germfree rats with the normal microbial flora a pronounced lag phase was noted before the gamma globulin level became normal. This lag phase was most pronounced in growing rats.

Newborn rats seem to start gamma globulin production more rapidly than older germfree rats. The response with regard to gamma globulin production on contamination of germfree rats with different types of bacterial cells through the natural routes is not identical.

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