GAMMA GLOBULINS IN GERM-FREE RATS*

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It is not known whether the gamma globulins in the blood of newborns are entirely of maternal origin. However, since gamma globulins and serum antibody normally decrease during the first 2 to 3 months of human life (1, 2) and since Good *et al.* (3) found a child borne by a mother with acquired agammaglobulinemia to have no gamma globulins until it was 1 month old, after which the gamma globulin concentration began gradually to rise, it may be assumed that a major part of the gamma globulins in the human newborn are derived from the mother. The initial postnatal decline in the concentration of the gamma globulins reaches a minimum about 2 to 3 months after birth, at which time the concentration is about one-third that of the normal adult value. Thereafter the level increases again, but does not approach the adult value until the child is about 2 years old (1, 2). It is not known whether this increase is caused by stimulation of endogenous or of exogenous factors.

The purpose of this investigation was to determine whether the normal bacterial flora in the body and in the environment might have something to do with the normal production of gamma globulins.

Material and Methods

The germ-free animals[1] were raised as described by Gustafsson (4) with slight modification of the rearing apparatus.

After having been weaned at 20 to 22 days the rats were fed diet D5 (Table I) with water *ad lib.* The diet was mixed with an equal volume of water and autoclaved at 121°C. for 20 minutes. The litters were born in cages with wood shavings as bedding material. After having been weaned the animals were kept in stainless steel cages with raised screens.

The germ-free rats as well as the controls were offspring of a male and a female from a strain of rats reared for many years in the Department of Histology. Thirty-four 50 to 150 day old animals of both sexes from the 2nd to 5th generations of the germ-free strain were studied. The controls were born and kept outside the apparatus and given the same sterilized diet D5. To evaluate the influence of the sterilization of the diet, the control material included rats fed on an unsterilized diet D5. The total number of controls was 36.

Twice a week feces and waste from the germ-free apparatus were studied for sterility.

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[1] Although called "germ-free" to conform to current usage, the animals actually can be considered only "bacteria-free," since viruses and rickettsiae were not excluded.

The following culture media were used under aerobic and anaerobic conditions: N.I.H.[2] thioglycollate broth, fluid thioglycollate medium, brain-liver heart medium, glucose broth, and Sabouraud's agar. Fecal smears were also studied microscopically.

At the end of the experiment the animals were anesthetized and bled. Blood was collected from the abdominal aorta, and allowed to clot spontaneously. Samples of serum for protein analysis were centrifuged at about 5000 *g* for 30 minutes to separate the chylomicrons.

The concentration of *total proteins* was determined in the earlier studies by the biuret

TABLE I

Diet D5

Casein	22 per cent
Wheat starch	63 " "
Arachis oil	10 " "
Salt mixture HMW*	4 " "
Vitamin mixtures	1 " "
Vitamins added per 100 gm. diet	
Vitamin A	2100 I.U.
Vitamin D	450 "
Vitamin E	50 mg.
Vitamin K	10 "
Thiamine	5 "
Riboflavin	2 "
Pyridoxin	2 "
Calcium pantothenate	10 "
Nicotinamide	20 "
Choline	200 "
Inositol	100 "
P-aminobenzoic acid	30 "
Biotin	0.1 mg.
Folic acid	2 mg.
Vitamin B_{12}	0.002 mg.
Ascorbic acid	100 mg.

* According to Hubbell, Mendel, and Wakeman (5).

method and later by Waddel's method (6). *Paper electrophoresis* was performed according to Laurell *et al.* (7). *Starch-gel electrophoresis* was carried out according to Smithies (8), the *zinc sulfate test* according to Kunkel (9), and *total lipides* according to Swahn (10).

RESULTS

The zinc sulfate test gave such low values for rat sera that even healthy rats with a normal concentration of gamma globulin fell within the range of human sera with so called agammaglobulinemia. The results were found to vary more with the opalescence of the serum and other factors than with the actual concentration of the gamma globulins. This test was therefore of no use for measuring the concentration of gamma globulins in rat sera.

[2] National Institutes of Health.

The total lipides varied between 250 and 3000 mg./100 ml. This was largely due to differences in the chylomicron content. However, since the protein content of the chylomicrons is very low (11), the distribution of the proteins did not vary with the degree of alimentary lipemia.

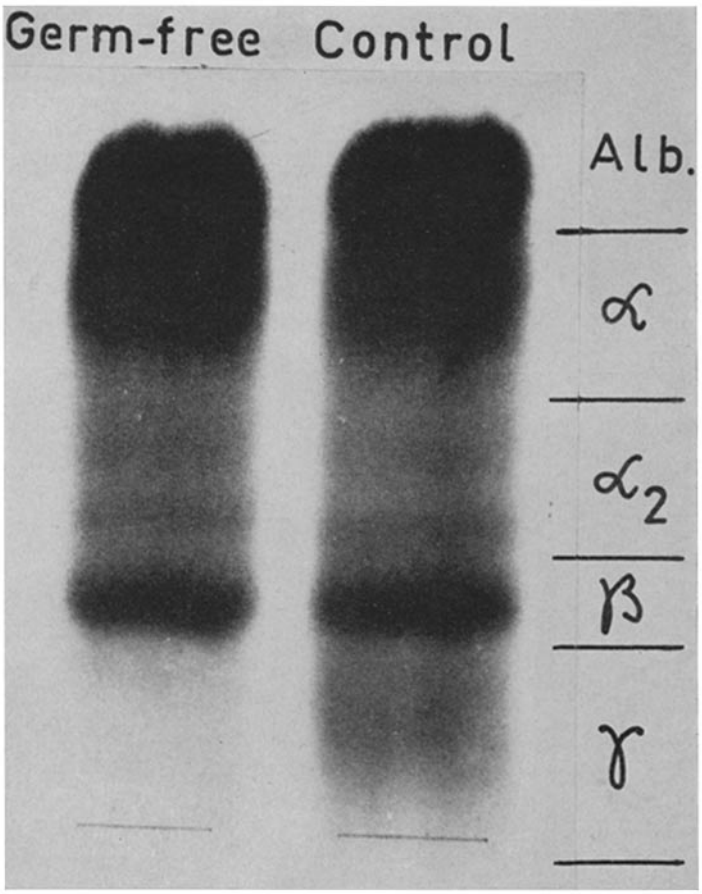

FIG. 1. Electrophoretic protein pattern

In the analysis of the electrophoretic diagrams we used a system resembling that employed in the classification of the electrophoretic fractions of human sera. Fig. 1 illustrates the method of subdividing the patterns. The sharp beta peak facilitated evaluation of the gamma fraction. The boundaries of the albumin fractions were also readily recognized. On the other hand, the limit lines of other fractions were more difficult to distinguish and this uncertainty decreased the accuracy of the method. A relatively large percentage of the serum

TABLE II

Serum Protein Values in Germ-Free and Control Animals of 2nd to 5th Generation

All values are expressed as gm. per 100 ml. serum.

	Serum protein		Albumin		Beta globulin		Gamma globulin			
							Observed values		Calculated values*	
	Germ-free	Controls	Germ-free	Controls	Germ-free	Controls	Germ-free	Controls	Germ-free	Controls
Mean value...	5.53	5.89	3.24	3.05	0.56	0.72	0.21	0.49	0.09	0.37
Maximum value.....	6.9	6.7	4.2	4.0	0.81	0.87	0.30	0.68	0.18	0.56
Minimum value.....	4.5	4.9	2.4	2.4	0.32	0.55	0.12	0.29	0.00	0.17
Standard errors of the mean.....	0.084	0.077	0.067	0.070	0.017	0.013	0.0062	0.018	0.0062	0.018
S.D.....	0.49	0.46	0.39	0.42	0.10	0.08	0.036	0.11	0.036	0.11
No.....	34	36	34	36	34	36	34	36	34	36

* For explanation see discussion.

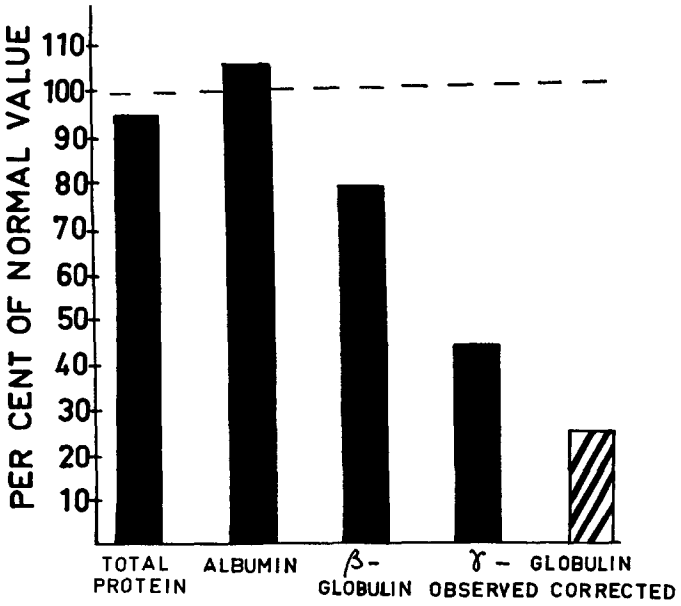

FIG. 2. Serum protein values of germ-free rats in per cent of those of control animals

samples contained hemoglobin in a concentration of 10 to 50 mg. per 100 ml.; as a result the variation of alpha$_2$ and beta components in the sera exceeded the normal biological range.

The data obtained were analyzed statistically (Table II). Since no differences

were found between the control animals on the sterilized diet and those on an unsterilized diet, the animals were considered together as a single group. Fig. 2 gives the concentrations of the different fractions as percentages of normal.

The concentrations of gamma globulin in the germ-free animals belonging to generations 2 to 5 are summarized in Table III, which also includes 8 rats of the first generation delivered by Caesarean section and hand-fed.

TABLE III

Gamma Globulin Values (Gm. per 100 Ml.) in Germ-Free Rats

Germ-free generation	No. of animals	Mean value	Range
1	8	0.26	0.45–0.12
2	3	0.25	0.30–0.19
3	13	0.20	0.26–0.17
4	10	0.20	0.25–0.16
5	8	0.22	0.29–0.15

DISCUSSION

The concentration of gamma globulin in the germ-free animals was found to be 0.21 ± 0.006 gm. per 100 ml. (Table II), as against 0.49 ± 0.018 in the control group. Corrected gamma globulin values are also given in the table.

Owing to trailing the electrophoretic gamma fraction also included material from other fractions. In order to correct for this inclusion, a constant factor, 0.12 gm. per 100 ml., was subtracted from the apparent gamma globulin values. This value (0.12) was selected since analyses of sera from patients with congenital "agammaglobulinemia" required a correction of this order. Since rat serum proteins differ somewhat in physical and chemical properties from human serum proteins, it is unlikely that the trailing is exactly the same for both types, but the corrected gamma globulin values are probably more accurate as semiquantitative measures of the true gamma globulin concentrations.

The gamma globulin concentration did not decrease from one germ-free generation to the next (Table III). However, the first generation of germ-free animals were hand-fed on an artificial milk formula and therefore were not strictly comparable to the subsequent generations.

As regards the beta fraction, there was also a statistically significant, although less striking difference between germ-free animals (0.56 ± 0.017 gm. per 100 ml.) and controls (0.72 ± 0.013). Interpretation of this difference is uncertain since electrophoretic separation of the gamma and beta fractions of rat serum is not as good as in human, horse, or rabbit serum. Immunological studies by Grabar (12) and others (13) have shown that although the bulk of the antibodies belong to the gamma fraction, the beta fraction also contains components that increase on antigenic stimulation and react with anti-gamma

globulin sera. The lower beta value in the germ-free animals might therefore be due to the same factors causing the low concentration of gamma globulin.

The "reticuloendothelial" cells have long been considered the main site of production of antibodies. In recent years strong support for this has been produced by studies of patients with so called congenital agammaglobulinemia in which highly sensitive and specific immunochemical methods (14, 3) have shown the gamma globulin concentration to be less than 2 per cent of normal. In these patients parenteral administration of antigens is not followed by the appearance of antibodies in the circulation and the reticular system including the plasma cells is markedly reduced (3).

To sum up, it has been shown that under the present experimental conditions hypogammaglobulinemia developed in rats which were reared in the absence of bacteria. The values did not, however, drop to such low levels as in congenital agammaglobulinemia in man. On the basis of our present knowledge of the various hypogammaglobulinemias in man we must consider two groups of possible causes:—

1. Normal synthesis of gamma globulins combined with abnormal losses (*e.g.* nephrotic syndrome).

2. Decreased synthesis due to (*a*) protein deficiency, (*b*) increased adrenal activity (oxysteroids), (*c*) decreased number of gamma globulin-producing cells (in congenital acquired hypo-agammaglobulinemia, myeloma, reticulosis, and sometimes in leukemias).

It seems highly probable that the hypogammaglobulinemia of germ-free rats was due to decreased synthesis since external loss can be excluded.

The problems in preparing an adequate diet for rearing germ-free animals are considerable; some diets might be inadequate for protein synthesis and thus depress production of gamma globulins. For example, Krebs (15) and others found hypogammaglobulinemia in patients with uncomplicated protein deficiency. But in the present investigation the germ-free rats did not differ from the controls in total serum protein concentration. Nor was any difference found in the concentration of albumin (Table II), a relatively sensitive indicator of pathological processes and of protein deficiency. In addition the growth curves for the germ-free animals and the controls were almost identical. There was thus no reason to assume that the nutrition was inadequate.

No signs of endocrine disorder were observed. Although the excretion of steroid hormones was not determined, the normal fertility of the animals argued against the existence of any serious endocrine disorder. Finally, histologic examination revealed no evidence of any of the diseases known to be accompanied by a decrease in the number of gamma globulin producing cells.

Assuming that the gamma globulins survive equally long in the circulation in germ-free animals as in controls, it may be concluded from the present results that gamma globulins are produced at a three times greater rate in normal than in germ-free animals. Recently Thorbecke *et al.* (16) presented data show-

ing that the concentration of gamma globulin in plasma of germ-free chickens (1st generation) older than 4 weeks was considerably lower than in the plasma of the controls.

This strongly suggests that the bacterial antigens obtained from the environment and from microorganisms harbored in the intestinal tract, the mouth, the respiratory tract and so forth, represent the normal, quantitatively dominant stimulus for gamma globulin–producing cells in mammals and birds. Whether antigenic stimuli are the only causes of gamma globulin production is still debatable. The question cannot be settled until animals can be reared not only bacteria free, but also without exposure to any other kind of antigen.

SUMMARY

The electrophoretic fractions of serum proteins were studied up to the 5th generation in a colony of germ-free rats. The germ-free animals had significantly lower concentrations of beta and the gamma globulins, while the other fractions were within normal limits.

Assuming that the gamma globulins survive equally long in the circulation in germ-free animals as in controls, the production of gamma globulins in normal rats is three times as rapid as in germ-free rats. This suggests that the normal flora of microorganisms is an important stimulant for the gamma globulin–producing cells.

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