THE LYMPHATIC SYSTEM OF GERMFREE GUINEA PIGS

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A germfree animal may be defined as one that has been born and raised under sterile conditions so that it has had no contact with bacteria and thus has never experienced any bacterial invasion of its tissues. It is a matter of great interest to determine the effects of such a germfree life on lymphatic tissues.

Methods

The guinea pigs that we use are of the Gifu uniform strain obtained by brother-sister breeding. We have obtained and reared germfree guinea pigs using the M-51 and M-56 germfree systems that I described previously[1] and elsewhere in this monograph. However, the rearing capacity of both of these systems is limited to 10 animals at one time, so that it is impossible to maintain a sufficient supply of animals more than 100 days old for the purpose of examining their lymphatic tissues. Consequently, we have reared the germfree animals until they are 40 to 50 days of age and then supplied several animals to several experimental groups, the results of which are supplemented by data obtained from an experiment on germfree animals more than 50 days old. Some of our germfree guinea pigs had a series of specific symptoms, such as alopecia, prolapsus ani, and hernia. These we consider due to the deficiency of essential nutritional elements, probably caused by a disturbance of ingestion and resorption in the bowel; therefore we have excluded these animals as unsuitable for the experiments. Kobayashi fluid diet NG-27 (TABLE 1) is given as soon as the young are born, on the fifth day after birth Kobayashi solid diet NG-36 (TABLE 2) is mixed with the diet NG-27, and at the end of the first month the NG-36 diet solely is used. NG-36 solid diet has been found satisfactory for the long-term rearing experiment, but NG-27 diet, a liquid formula for young guinea pigs, still needs improvement before it can be used satisfactorily. Therefore a comparison has been made with respect to the growth within 20 days of birth between germfree guinea pigs reared on the Kobayashi diet and natural guinea pigs reared with the same diet. The result indicated that the germfree animals grow more slowly than those reared in a natural environment. After 30 days of age the average increasing rate of body weight per day of the germfree animal becomes parallel with that of the natural animals reared on the Kobayashi diet. Because there was a handicap of growth during the first 20 days of life, the body weight of the germfree animals is always less than that of normal animals of the same age. Keeping this fact in mind, we have made efforts to reduce the body weight of the control animals to the level of the germfree ones. Both germfree and control animals were kept in the same environmental conditions, that is, all experimental animals were reared at a temperature of 25° ± 3° C. and in the humidity range of 50 ± 5 per cent. We also used a considerable number of natural animals as a preliminary experiment, as well as the paired germfree and control animals.

TABLE 1

COMPOSITION OF NG-27 DIET (KOBAYASHI) PER 300 GM. OF RATION

Whole dry milk	100 gm.
Aq. dest.	200 ml.
Soy beam oil	1.5 ml.
Panvitan (vitamin complex)	3 pellets
Folic acid	7.5 mg.
Vitamin K	7.5 mg.
Vitamin B	30.0 μg.
Biotin	300.0 μg.
Choline	0.15 gm.
Yeast extract	1.5 gm.
Liver extract	75.0 mg.
Inositol	300.0 mg.
PABA	15.0 mg.
Thiamine	27.0 mg.
Riboflavin	6.0 mg.
Pyridoxine	6.0 mg.
Vitamin C	20 to 25 mg./2 days

COMPOSITION OF NG-36 DIET (KOBAYASHI) PER 100 GM. OF RATION

Roasted soy bean flour	70.0 gm.
Sucrose	9.0 gm.
Gum arabic	12.0 gm.
Salts mix	7.0 gm.
Panvitan (vitamin complex)	1 pellet
Folic acid	2.5 mg.
Vitamin K	2.5 mg.
Vitamin B	15.0 μg.
Biotin	100.0 μg.
Choline	0.3 gm.
Yeast extract	0.5 gm.
Liver extract	25.0 mg.
Inositol	100.0 mg.
Pantothenic acid-Ca	10.0 mg.
PABA	5.0 mg.
Thiamine	9.0 mg.
Riboflavin	2.0 mg.
Pyridoxine	2.0 mg.
Vitamin C	25 mg./2 days

Results

The experimental results were as follows.

First, we made a preliminary investigation on many conventional animals, ranging in development from fetus to adult, in order to discover the essential states of the lymphatic apparatus in the nasal cavity. For this purpose the examinations were made by serial frontal section of 50 animals and sagittal section of 4 animals. The results reveal the fact that the anatomical structure of the nasal cavity in the guinea pigs is complicated. A prenatally formed lymphatic apparatus on the central line of the floor of the nasal cavity extends from the posterior edge of the foramina palatina to the position of the fourth molar. Most of the lymphatic tissue contains postnatally formed secondary nodules with clear centers designated by Hellman[2] as *Reactionszentrum*. For instance, in the 40-day-old conventional guinea pig, well-developed lymphatic tissue was found in the nasal cavity, wherein not only solid secondary nodules but also clear-centered secondary nodules, called Flemming-type nodules,[3] were formed. The ciliated epithelium in the mucous membrane became stratified (FIGURES 1 to 4). In germfree guinea pigs of the same age, similar lymphatic tissue and occasional solid secondary nodules were found at the nasal floor, but no clear-centered nodules (FIGURES 1, 2, 5, and 6). In an attempt to investigate the lymphatic tissue in the sinus maxillaris, this cavity was examined in 30 natural animals ranging in development from fetus to adult, serial section specimens being made. The results of the examination revealed that the sinus maxillaris of the guinea pig is a complicated cavity that is not completely encircled by bone, but is open on both sides. As FIGURE 7 shows, distinct secondary nodules were formed in the tunica propria of the mucous membrane of the sinus maxillaris in 40-day-old control animals, and clear centers were recognizable in the nodules. The ciliated epithelium of the mucous membrane of sinus maxillaris has also become stratified. However, in germfree animals of the same age neither secondary nodules nor even lymphocytic infiltrations were found in the tunica propria of the sinus maxillaris; therefore a monotonous picture is seen. The ciliated epithelium covering the sinus maxillaris was arranged regularly in a single layer (FIGURE 8).

Second, we examined the number of Peyer's patches in the cecum of the following 103 natural animals: 50 cases from birth to 20 days of age, 10 cases about 40 days of age, and 43 cases 150 to 200 days of age. It was revealed that 6 to 25 patches were observed in the young animals shortly after birth, 8 to 15 in 40-day-old animals, and 8 to 16 in the 150- to 200-day-old animals. The maximum number of patches in each group was 12 in the young animals, 11 or 12 in the 40-day-old ones, and 13 in the 150- to 200-day-old animals; thus, it ranged from 11 to 13. This fact indicates that the number of Peyer's patches is settled before birth.[4, 5] However, the examination of 17 cases of more than 40-day-old germfree guinea pigs has shown the number of Peyer's patches to be from 6 to 8. However, it is not clear whether the smaller number has any significant meaning. Histologically, only a few secondary nodules with clear centers were found in the Peyer's patches in the cecum of the natural young shortly after birth, but great numbers of the nodules were found in the 40- to 200-day-old natural animals. For instance, in the Peyer's patches of a 40-day-old control guinea pig one can see that a distinct and clearly bordered secondary nodule with clear center has appeared (FIGURE 9). On the other hand, in the Peyer's patches of a 40-day-old germfree guinea pig, as may be seen in FIGURE 10, secondary nodules with clear centers were not found, and the Peyer's patch itself is very poorly developed.

Third, in regard to lymph nodes, a preliminary investigation has been made on these nodes in popliteal, abdominoinguinal, mesenteric, and cecal areas of 50 of the young animals shortly after birth, 9 of 40- to 50-day-old ones, and 39 of 150- to 200-day-old animals. As result of the examination of the lymph nodes of 211 germfree animals we can say that the lymph nodes in germfree animals develop more poorly than do those in natural guinea pigs. It is con-

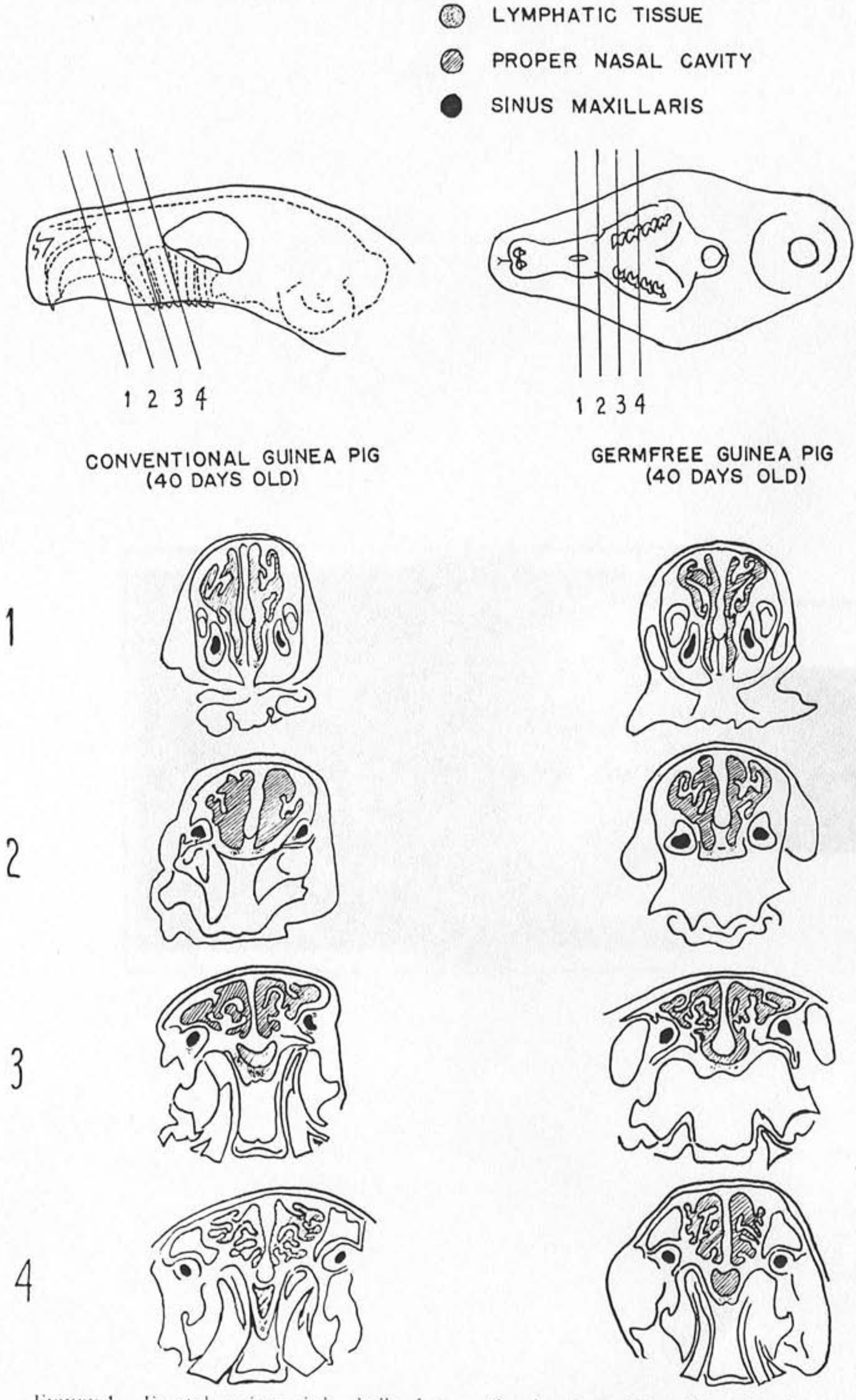

FIGURE 1. Frontal sections of the skulls of conventional and germfree guinea pigs, showing the lymphatic tissue of the nasal cavity and the sinus maxillaris.

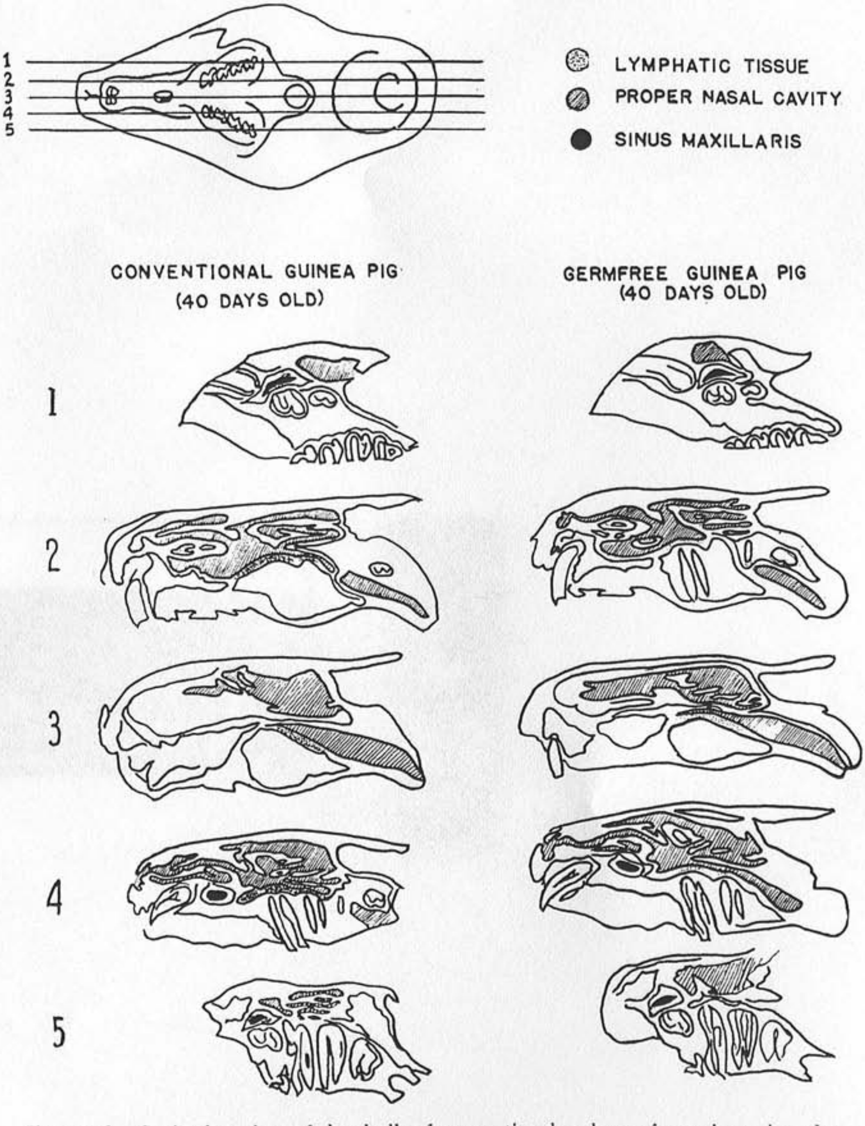

FIGURE 2. Sagittal sections of the skulls of conventional and germfree guinea pigs, showing the lymphatic tissue of the nasal cavity and the sinus maxillaris.

sidered that the mesenteric and cecal lymph nodes have different functions in relation to the intestinal tracts. Even though they are germfree, these animals could not be exempted from the influence of stimulation originating from food in the bowel. Histologically, we could recognize solid secondary nodules, but few secondary nodules with clear centers in the natural guinea pig shortly

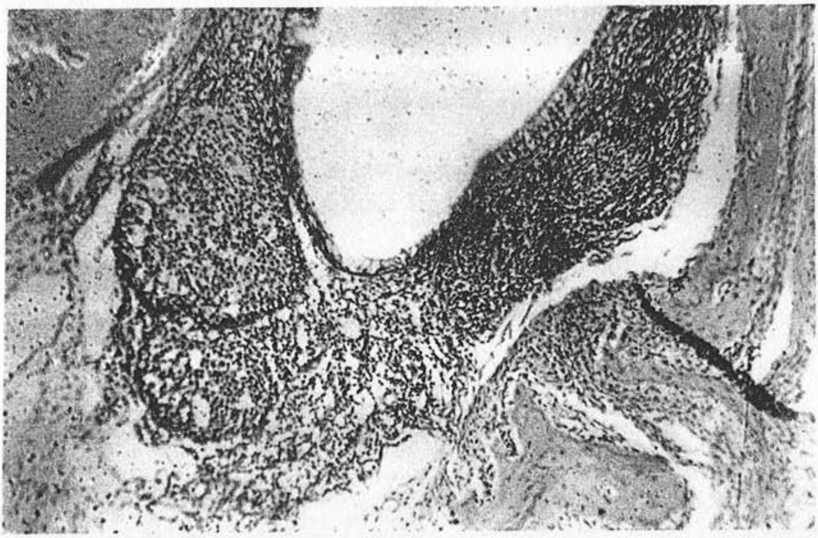

FIGURE 3. Frontal section of the skull of a conventional guinea pig. Note the well-developed lymphatic tissue in the tunica propria of the nasal cavity. Distinct clear-centered secondary nodules are visible. Hematoxylin-eosin stain. ×280.

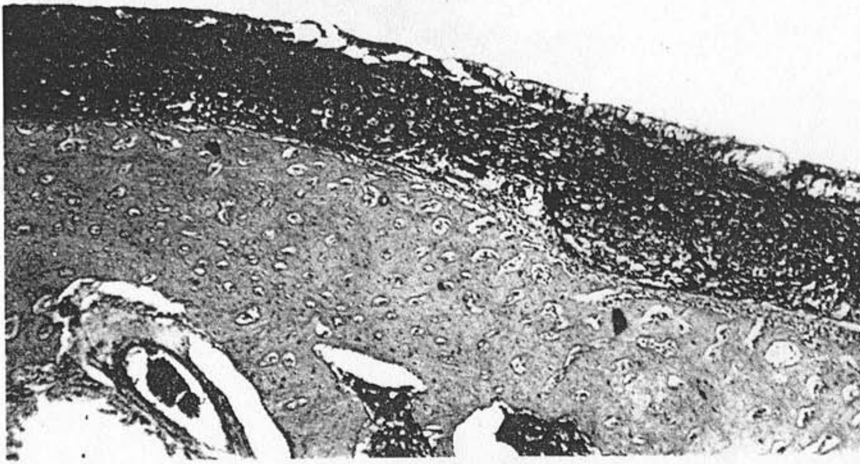

FIGURE 4. Sagittal section of the skull of a conventional guinea pig. Note the well-developed lymphatic tissue in the tunica propria of the nasal cavity. Clear-centered secondary nodules, as well as solid ones, are visible. Hematoxylin-eosin stain. ×280.

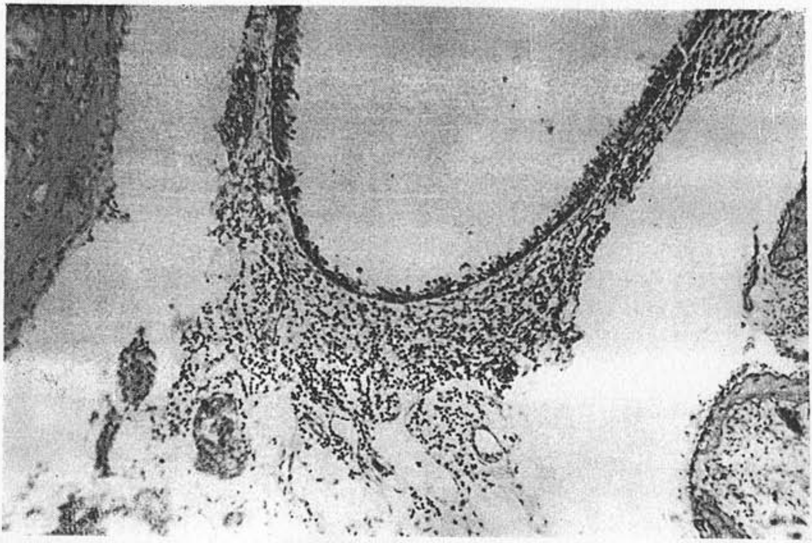

FIGURE 5. Frontal section of the skull of a germfree guinea pig. Note the poorly developed lymphatic tissue in the lamina propria of the nasal cavity. No clear-centered nodules are visible. Hematoxylin-eosin stain. ×280.

FIGURE 6. Sagittal section of the skull of a germfree guinea pig. Note the poorly developed lymphatic tissue in the tunica propria of the nasal cavity. No clear-centered nodules are visible. Hematoxylin-eosin stain. ×280.

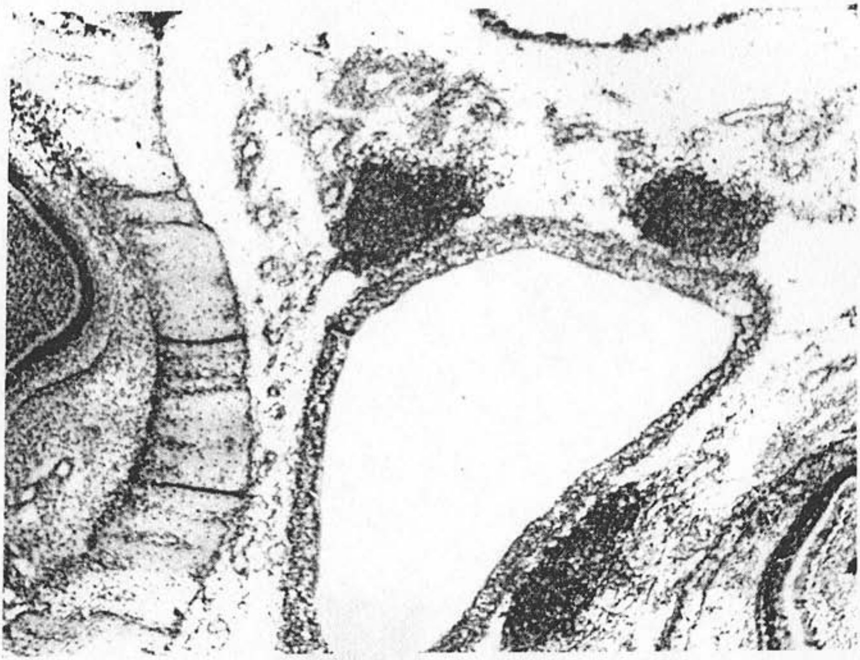

FIGURE 7. Frontal section of the skull of a conventional guinea pig, showing distinct secondary nodules in the tunica propria of the mucous membrane of the sinus maxillaris. Note the clear-centered nodules. Hematoxylin-eosin stain. ×280.

FIGURE 8. Frontal section of the skull of a germfree guinea pig. Neither secondary nodules nor even lymphatic infiltration are visible in the tunica propria of the sinus maxillaris. Hematoxylin-eosin stain. ×280.

FIGURE 9. The cecum of a 40-day-old conventional guinea pig. Note the well-developed Peyer's patch and the clear-centered nodules. Hematoxylin-eosin stain. ×280.

after birth. However, in the 40- to 200-day-old animals we could find numerous clear-centered secondary nodules, particularly in the mesenteric and cecal lymph nodes. In the 40- to 200-day-old animals, some of the cases have shown that pyronin-stained large cells and typical Marschalko-type plasma cells appear in the medullary cords. For instance, in the popliteal lymph node of a 40-day-old natural control guinea pig, not only solid secondary nodules but also clear-centered secondary nodules on the cortex and numerous pyronin-stained large cells and typical plasma cells on the medullary cord were observed (FIGURE 11). On the other hand, in the popliteal lymph node of a 40-day-old germfree guinea pig, no clear-centered secondary nodules were found, but plasma cells appeared on the medullary cord (FIGURE 12).

Let us now consider the behavior of the lymph nodes in the cases contaminated by single species of bacteria. We have made an investigation of 19 guinea pigs that were monocontaminated because of a failure in the germfree unit during the rearing process. Nine of these animals were contaminated by a single species of spore; the other 10 were contaminated by a single species of *Coccus*. Five succumbed to a bloody watery diarrhea and, in 3 of the 5 deaths, similar involvement was observed in the cecum. Macroscopically, emphysema, necrosis, and bleeding were observed; microscopically, the breakdown of Peyer's

FIGURE 10. The cecum of a 40 day-old germfree guinea pig. Note the poorly developed Peyer's patches and the absence of clear-centered nodules. Hematoxylin-eosin stain. ×280.

patches into numerous small pieces by air vesicles was seen in the wall of the cecum (FIGURE 13). Secondary nodules with clear centers were also found in the cecal lymph nodes (FIGURE 14). Numerous large-sized cells with large nucleoli were observed by making stamp specimens of the node. However, the origin of the large cells cannot be determined. Marschalko-type plasma cells were often detectable on the stamp specimen of the lymph nodes of the germfree animals, as shown in FIGURE 15. However, no abnormalities occurred in the conventional animals that were challenged by the same single species of

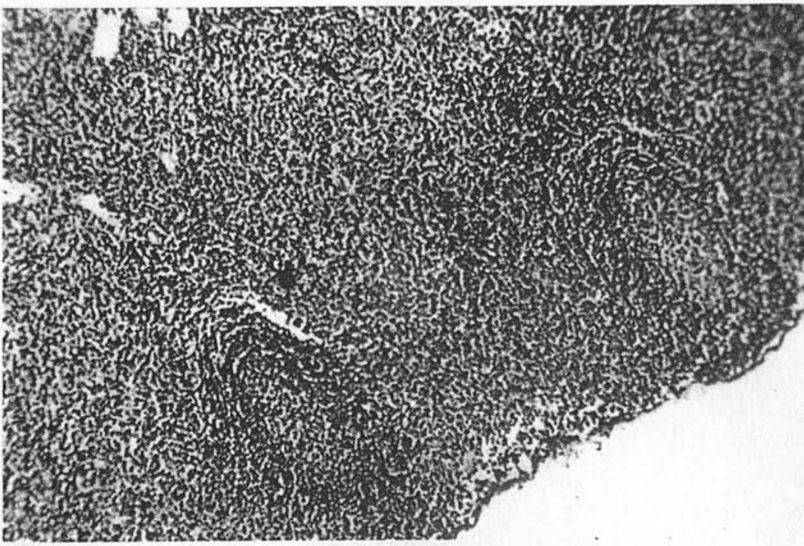

FIGURE 11. Popliteal lymph nodes of a 40-day-old conventional guinea pig. Note the solid and clear-centered nodules. Hematoxylin-eosin stain. ×280.

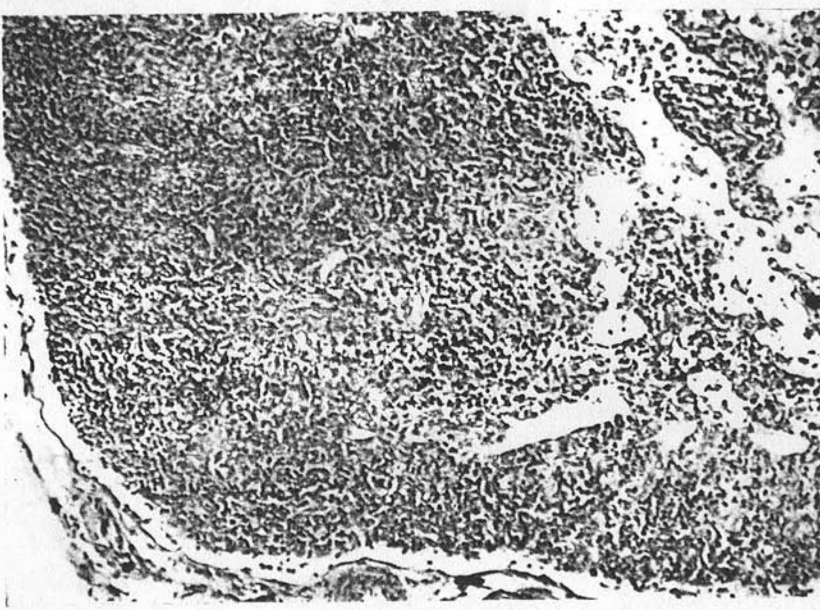

FIGURE 12. Popliteal lymph node of a 40-day-old germfree guinea pig. Hematoxylin-eosin stain. ×280.

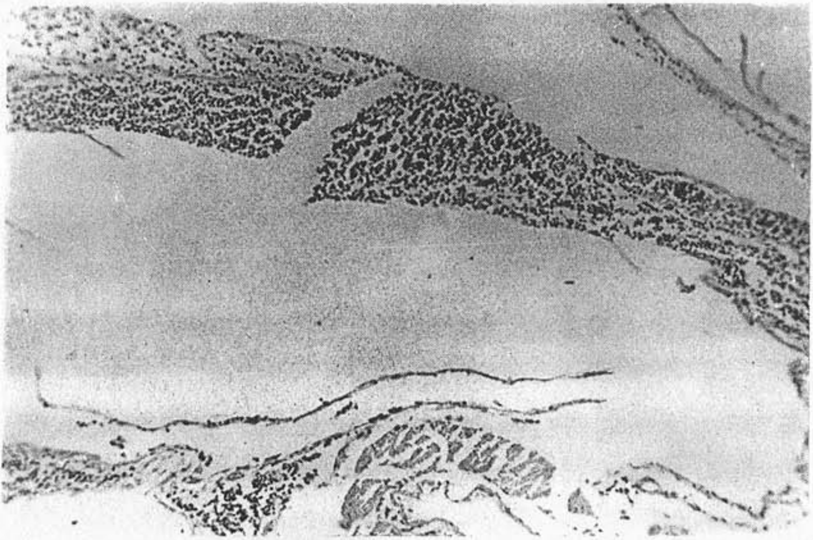

FIGURE 13. Cecum of a germfree guinea pig contaminated by a single species of spore. Note the breakdown of the Peyer's patch into small fragments by air vesicles. Hematoxylin-eosin stain. ×280.

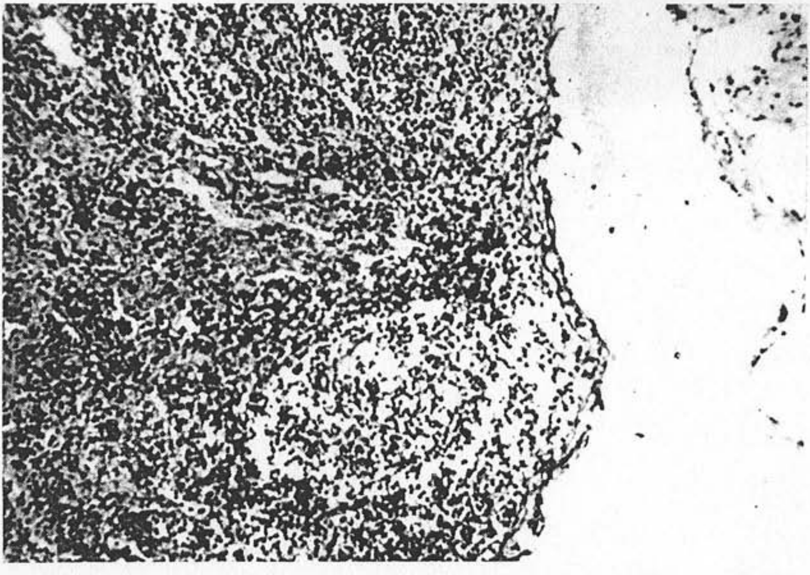

FIGURE 14. Cecal lymph node of a germfree guinea pig contaminated by a single species of spore. Note the appearance of clear-centered nodules. Hematoxylin-eosin stain. ×280.

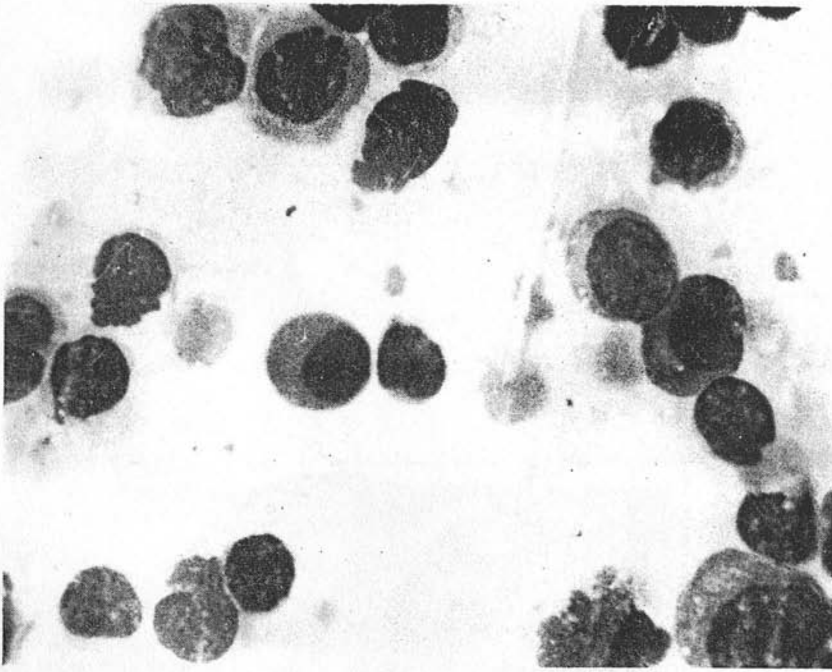

FIGURE 15. Stamp specimen of the cecal lymph node of a germfree guinea pig contaminated by a single species of spore. Note Marschalko's plasma cells. Hematoxylin-eosin stain. ×900.

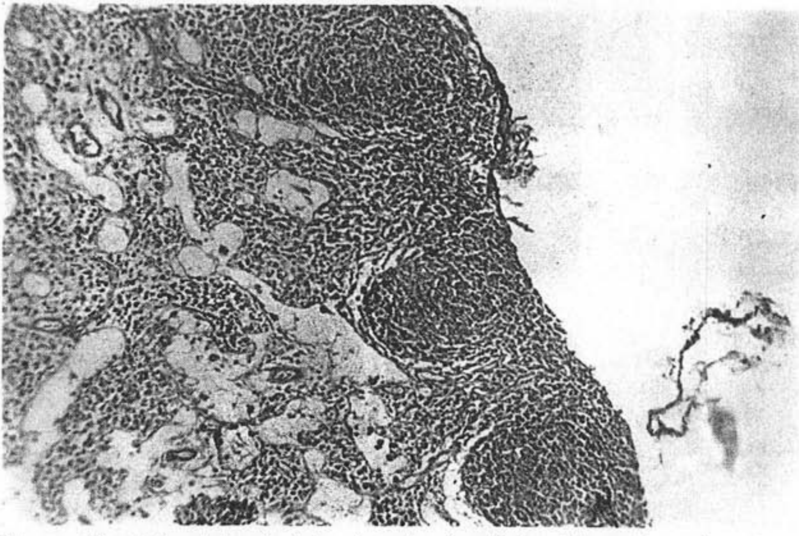

FIGURE 16. Abdominoinguinal lymph node of a 40-day-old germfree guinea pig contaminated by a mild species of *Coccus*. Note the bare, clear centers without peripheral dark rings. Hematoxylin-eosin stain. ×280.

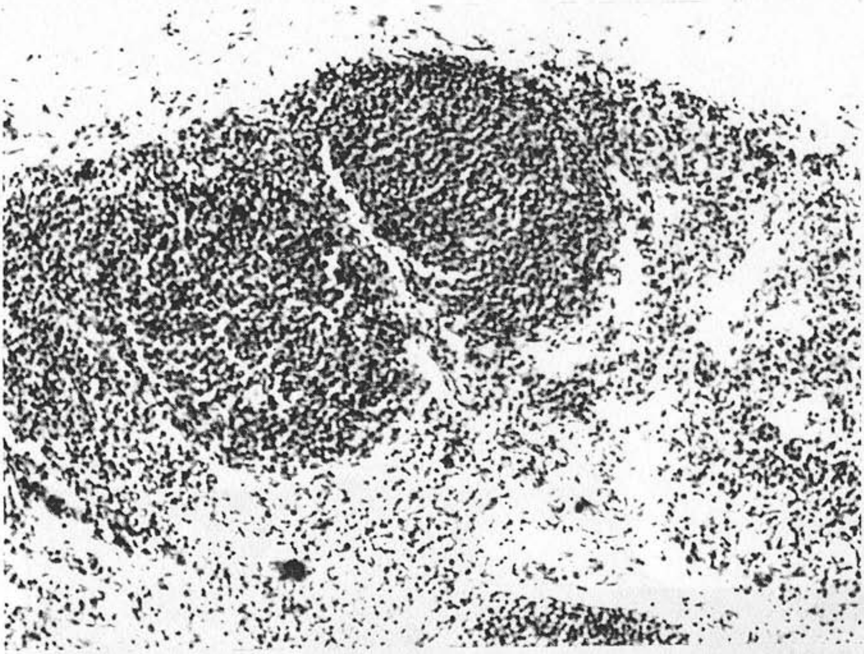

FIGURE 17. Popliteal lymph node of a conventional guinea pig that had been injected in the foot pad with 16 mg. sterilized egg albumin. Distinct clear-centered nodules are visible. Hematoxylin-eosin stain. ×280.

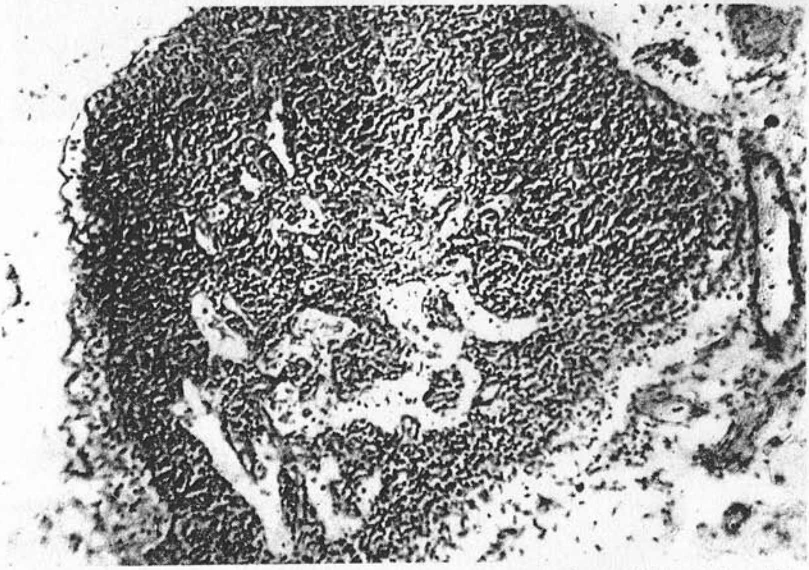

FIGURE 18. Popliteal lymph node of a germfree guinea pig that had been injected in the foot pad with 10 mg. sterilized egg albumin. Hematoxylin-eosin stain. ×400.

spore. It seemed to us that the result described above has indicated the existence of an antagonism between certain microbes in the natural world; in other words, certain bacteria that do not cause pathogenicity under the antagonisms in the natural conventional animal appear to cause pathogenicity in a monocontaminated germfree animal. An investigation of the lymph nodes in the germfree guinea pigs monocontaminated by mildly virulent *Coccus* has revealed that numerous secondary nodules with clear centers and without distinct dark rings appeared. For instance, in the abdominoinguinal lymph node of the 40-day-old germfree guinea pigs contaminated by a mild species of *Coccus*, numerous bare clear centers without peripheral dark rings were apparent. They are like those described as bare germinal centers by Conway[6] (FIGURE 16). Consequently, the results of the experiment indicate that some contamination by mildly toxic bacteria such as cocci contributes essentially to the production of the clear center in the lymphatic tissue. Next, we examined the lymph nodes of germfree animals injected with sterilized egg albumin. Preceding this observation, a preliminary investigation of the changes in lymph nodes of the 66 conventional guinea pigs injected with egg albumin was made; this revealed the fact that the clear-centered secondary nodules on the cortex increased in number and that plasma cells on the medullary cord were prominent on the injected side on the sixth to tenth day after injection as compared with the noninjected side when the precipitation reaction was positive. For instance, 40-day-old conventional guinea pigs that have been injected with 10 mg. of sterilized egg albumin in the foot pads have shown distinct secondary nodules with clear centers and dark rings in the popliteal lymph node in 48 hours (FIGURE 17). After a similar procedure, 40-day-old germfree guinea pigs have shown no nodules with clear centers, but numerous large pyronin-stained cells of diffuse appearance with a large nucleolus that contains ribonucleic acid have been observed. It would be interesting to learn whether these cells gather together to form a nodule (FIGURE 18).

Conclusion

We have thus learned that the development of lymphatic tissue in the germfree animal is less advanced than in the conventional animal. Insofar as we have been able to discover, typical clear-centered nodules have never been found in the lymphatic tissue of germfree animals, but such nodules have been produced most easily by monocontamination by a mildly toxic species of *Coccus*. Since lymphatic tissue is widely considered as the main site of antibody formation,[7-10] the gamma-globulin value of the serum and the state of antibodies such as precipitin and agglutinin in the treated germfree animal are of definite interest.

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