Serum β-Lysin and Muramidase Levels in Germfree and Conventional Rats (34404)

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β-Lysin and muramidase (lysozyme), bactericidal substances found in serum and in other tissues, presumably play a role in the hosts' nonspecific defense mechanism (1–3). The primary source of serum β-lysin is the platelet (4) which ruptures during the coagulation process. Most of the muramidase in serum is attributable to release from leukocyte disruption and autolysis (2, 5). It was felt that a study of these two antibacterial activities in serum from germfree animals might prove interesting because of the deficiency in various lymphoid elements (6, 7) with accompanying low antibody (8, 9) and immunoglobulin (9, 10) levels in these types of animals. In regard to extracellular β-lysin, we are not aware of any reports on comparative platelet counts between germfree and conventional animals or of any significant differences in the coagulation process between these two animal groups. However, in reference to extracellular muramidase, reduced white blood cell counts in germfree species have been reported (11, 12). The present study was therefore designed to determine β-lysin and muramidase activity in the serum of germfree (GF) rats and to compare the levels with those found in ex-germfree (exGF) and conventional (CV) rats of the same strain.

Methods and Procedures. Animals. Forty Sprague-Dawley GF weanling rats, equally divided as to sex, were obtained in a sterile transfer compartment from the Germfree Unit, Laboratory Aids Branch, NIH. Ten male and 10 female weanlings were transferred to a sterile Reyniers isolator and the remaining 20 were distributed into cages and placed in quarters generally used to house conventionally reared animals. The germfree weanlings were fed a steam-sterilized semisynthetic diet, L-356 (13). At 12 weeks of age, they were exsanguinated, leukocyte counts were made, and serum samples were obtained for appropriate analyses. The 20 GF weanlings, which were placed in the same room containing conventionally reared animals, were also fed diet L-356 and likewise exsanguinated at 12 weeks of age. These animals will be referred to as the exGF group. Twenty CV Sprague-Dawley weanling rats were obtained from the Rabbit and Rodent Production Section, NIH, at the same time as the GF group was received. These animals were fed commercial Purina pellets and then exsanguinated at 12 weeks of age.

β-Lysin activity in serum was determined by its bactericidal effect on a standard spore suspension of *Bacillus subtilis* (14). Viable cell counts were made following a 2-hr incubation period with serum at 37°. A unit of β-lysin activity was defined as that amount of serum required to kill 99% of the bacteria added to the reaction mixture.

The separation and purification of β-lysin was conducted as previously reported (15, 16), in which serum was first filtered through a Seitz asbestos-cellulose (type ST) filter followed by elution of the adsorbed activity from the inverted filter pad with 1.5 *M* NaCl solution. Overnight dialysis of the eluate against distilled water at 5° removed excess salt which is inhibitory to β-lysin activity. The supernate obtained after centrifugation for 15 min at 19,620*g* was then re-centrifuged

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TABLE I. β-Lysin in Serum of Conventional (CV), Ex-germfree (exGF) and Germfree (GF) Rats.

Animal group	Sex	No. of animals with titer				Total no.	Mean geometric titer	Combined mean
		64	128	256	512			
CV	M		1	3	5	10	315	
	F		1	3	6	10	362	338
exGF	M			4	7	11	398	
	F		2	7		9	219	304
GF	M		2	3	4	9	299	
	F	1	2	3	5	11	273	284

for 18–24 hr at 5° at 78,480*g*. The resultant precipitate which contained most of the bactericidal activity was resuspended in 10 times its volume of saline. By these procedures it was possible to recover most of the β-lysin activity originally present in serum.

Serum muramidase was assayed by measuring the reduction in turbidity of a standard suspension of *Micrococcus lysodeikticus* at 650 mμ using 3× crystallized egg white muramidase (Sigma Chemical Co.) as the enzyme standard (17). Total white blood cell and differential counts were determined by routine hematologic procedures.

Results. Following the separation and purification of β-lysin from a pool of germfree rat sera and assay of the various fractions obtained, the following differences in activity were noted which were not observed with serum samples obtained from conventionally reared rats: the percentage of bactericidal activity retained by Seitz-filtered germfree serum was twice as high as that seen with conventional serum: the bactericidal activity of the fraction eluted from the filter pad with 1.5 *M* NaCl was less than that of the original serum, although serum obtained from conventional rats showed twice as much activity in the elutate as in the original serum; the degree of purification of β-lysin from germfree serum was approximately 12-fold in contrast to a 100-fold purification achieved from conventional rat serum. Since blood platelets appear to be the major source of extracellular β-lysin in serum (2), the above differences might be due to differences in the numbers of platelets in blood between these groups of rats.

Comparative titers of β-lysin in the three animal groups are shown in Table I. A comparison of mean geometric titers using the Yates mean score test (18) shows neither a significant difference between the CV and GF group ($p>0.20$) nor between males and females within the CV ($p>0.20$) and GF ($p>0.20$) groups. However, a significant difference was found between exGF males and exGF females ($p<0.01$). Table I shows that the mean titer of β-lysin for exGF males is very similar to the levels found in CV animals; however, the mean β-lysin titer for exGF females approximates the somewhat lower GF levels.

A comparison of mean levels of muramidase between male and female rats of each group (Table II) showed significant differences only within the exGF group ($p<0.01$). The data shows that exGF male rats have higher muramidase levels than exGF females. It will be recalled that exGF male rats also had higher β-lysin activity than exGF females. When the mean levels of muramidase between the three groups were compared, CV rats had significantly higher levels of enzyme than exGF ($p<0.01$) or GF ($p<0.01$) animals. The exGF male rats had somewhat higher levels than GF ($p<0.01$) but lower than CV ($p<0.01$).

Since relatively high concentrations of muramidase are found in polymorphonuclear leukocytes and monocytes (19, 20) and it has been reported (5, 21, 22) that there is a positive correlation between muramidase levels and the number of granulocytes, we attempted to determine whether the differences noted with respect to muramidase

TABLE II. Muramidase Levels and Granulocyte Counts in Conventional (CV), Ex-germfree (exGF) and Germfree (GF) Rats.

Animal group	Sex	No. of sera	Muramidase (μg/ml) Range	Mean[a]	Combined mean	SE	No. of sera	Granulocyte count Mean[a]	SE
CV	M	8	11.0–14.8	12.9		0.5	10	2022	344
	F	8	10.6–14.2	12.3	12.6	0.4	10	1784	264
exGF	M	9	9.7–12.9	11.0		0.3	9	1015	99
	F	7	6.8–12.0	8.6	9.9	0.7	9	939	139
GF	M	7	7.7– 9.1	8.5		0.2	10	717	100
	F	9	7.1–12.5	10.0	9.3	0.5	4	556	105

[a] The Spearman rank correlation test was applied to the mean muramidase levels and the mean granulocyte counts: $r = +0.829$, $p = 0.03$.

levels might be related to differences in the number of granulocytes present in the groups. As expected (Table III), the number of circulating leukocytes are lowest in the GF, somewhat higher in the exGF and highest in the VC group. A similar trend is shown with respect to the mean granulocyte counts among the three groups (Table II). Comparison of the means of muramidase levels and number of granulocytes shows that these two sets of values are positively correlated, *i.e.*, high miramidase levels tend to be associated with high granulocyte counts and low muramidase levels, with low granulocyte counts.

Discussion. Numerous other studies of germfree animals have reported abnormally low or nonexistent levels of mucosal enzymes (23), β-glucosidase (24), lysozyme, acid phosphatase, and cathepsin in alveolar macrophages (25). The findings of low levels of antibodies and immunoglobulins in germfree species have been alluded to earlier in this report. Despite these aforementioned deficiencies, it has been reported, at least in regard to germfree rats (26), that overall protein synthesis by the spleen does not appear to be reduced.

Although no significant difference was noted in the β-lysin activity of CV and GF serum, re-assay of the various fractions obtained from GF serum purification showed considerable deviations from comparable fractions obtained from CV serum. Some of the underlying reasons for these differences might be clarified by further studies of the blood platelets, their extracts and the serum β-lysin component 1 of these two groups of rats. The case for low muramidase levels in the GF rat can be attributed, ultimately, to

TABLE III. White Blood Cell Types and Total Counts in Conventional (CV), Ex-germfree (exGF) and Germfree (GF) Rats.

Animal group	Sex	No. of animals	Percentage of cell types[a] L	N	M	E	B	Range of total count	Mean	Mean of both sexes
CV	M	10	70–90	7–22	0–15	0–2	0	11,100–23,150	17,360	
	F	10	66–91	6–24	1–23	0–2	0–1	9100–17,850	12,930	15,145
exGF	M	9	71–92	6–14	0–19	0–2	0	6100–13,900	10,699	
	F	9	75–97	3–22	0–3	0–1	0–1	4800–8375	6555	8627
GF	M	10	79–94	4–14	0–8	0–4	0	4350–9000	6670	
	F	7	79–92	4–16	0–4	0–1	0	2900–7700	5600	6229

[a] L, lymphocytes; N, neutrophils; M, monocytes; E, eosinophils; B, basophils.

reduced lymphoid development as a consequence of the germfree state.

A significant characteristic which distinguished one animal group from the other, in the three groups we have studied, was the qualitative and quantitative difference in the indigenous microbial flora. For example, the CV rat gastrointestinal normal flora was composed of large numbers of a wide range of aerobic and anaerobic microorganisms. On the other hand, our exGF group possessed a limited low-density, predominantly aerobic intestinal flora presumably as a result of their maintenance on a sterile semisynthetic diet. Our exGF group is, therefore, similar, if not identical to cesarean-derived, specific-pathogen-free (SPF) animals which are also characterized by a controlled, low-density microbial flora. SPF animals are raised under strictly controlled environmental conditions and reputed to be more uniform in their responses to experimental manipulation. However, the disparity of both β-lysin and muramidase levels between our exGF male and female rats suggests a closer examination of other parameters in regard to these sex differences in SPF animals.

Summary. A comparative study of serum β-lysin and muramidase activity in germfree, ex-germfree and conventional rats showed no significant differences in β-lysin levels between the three groups; however, upon separation and purification of β-lysin from germfree serum, unusual differences in the bactericidal activity of the different fractions obtained were noted. The CV rats showed significantly higher levels of muramidase than exGF or GF rats and these differences were closely correlated with differences in blood granulocyte counts. Higher levels of both β-lysin and muramidase were found in exGF male rats in contrast to exGF females.

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1. Skarnes, R. C. and Watson, D. W., Bacteriol. Rev. **21**, 273 (1957).

2. Jensen, R. S., Tew, J. G., and Donaldson, D. M., Proc. Soc. Exptl. Biol. Med. **124**, 545 (1967).

3. Tew, J. G., Hess, W. M., and Donaldson, D. M., J. Immunol. **102**, 743 (1969).

4. Donaldson, D. M., Jensen, R. S., Jensen, B. M., and Matheson, A., J. Bacteriol. **88**, 1049 (1964).

5. Finch, S. C., Lamphere, J. P., and Jablon, S., Yale J. Biol. Med. **36**, 350 (1964).

6. Luckey, T. D., "Germfree Life and Gnotobiology," p. 360 Academic Press, New York (1963).

7. Bauer, H., *in* "The Germfree Animal in Research" (M. E. Coates, ed.), p. 210. Academic Press, New York (1968).

8. Luckey, T. D., "Germfree Life and Gnotobiology," p. 370. Academic Press, New York (1963).

9. Ikari, N. S., Nature **202**, 879 (1964).

10. Asofsky, R., Ikari, N. S., and Hylton, M. B., *in* "Advances in Germfree Research and Gnotobiology" (M. Miyakawa and T. D. Luckey, eds.), p. 219. Chemical Rubber Co., Cleveland, Ohio (1967).

11. Abrams, G. D. and Bishop, J. E., Arch. Pathol. **79**, 213 (1965).

12. Caster, P., Garner, R. and Luckey, T. D., Nature **209**, 1202 (1966).

13. Larner, J. and Gillespie, R. E., J. Biol. Chem. **225**, 279 (1957)

14. Donaldson, D. M., Jensen, R. S., Jensen, B. M., and Matheson, A., J. Bacteriol. **88**, 1049 (1964).

15. Donaldson, D. M., Ellsworth, B., and Matheson, A., J. Immunol. **92**, 896 (1964).

16. Matheson, A., Jensen, R. S., and Donaldson, D. M., J. Immunol. **96**, 885 (1966).

17. Jolles, P., Methods Enzymol. **5**, 137 (1962).

18. Cochran, W. G., Biometrics **10**, 417 (1954).

19. Barnes, J. M., Brit. J. Exptl. Pathol. **21**, 264 (1940).

20. Flanagan, P. and Lonetti, F., Blood **10**, 497 (1955).

21. Hiatt, R. B., Engle, C., Flood, C., and Karusa, A., J. Clin. Invest. **31**, 721 (1952).

22. Jolles, P., Sternberg, M., and Mathe, G., Israel J. Med. Sci. **1**, 445 (1965).

23. Jerves, H. R. and Biggers, D. C., Anat. Record **148**, 591 (1964).

24. Spatz, M., McDaniel, E. G., and Laqueur, G. L., Proc. Soc. Exptl. Biol. Med. **121**, 417 (1966).

25. Heise, E. R. and Myrvik, Q. N., Federation Proc. **25**, 439 (1966).

26. Talal, N., Federation Proc. **25**, 439 (1966).

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