The Nature of Alpha Toxin Production by *Staphylococcus aureus* Grown *in vivo*.* (30102)

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The studies of Gladstone and Glencross(1) indicated that *Staphylococcus aureus* could readily multiply and elaborate a number of soluble components when grown within dialysis sacs implanted in the peritoneal cavity of certain animal species. While investigating various parameters involved in the intraperitoneal infection of mice with *S. aureus*, we had the opportunity to employ the above technic. The initial observations of Gladstone and Glencross have been confirmed by these studies. However, certain other observations, reported here, are concerned with the production of alpha toxin by a strain of *S. aureus* under these *in vivo* conditions.

Materials and methods. The data were obtained by using *S. aureus* 18Z(2), grown in trypticase soy broth with aeration and harvested in the late log phase. After centrifugation, the staphylococci were washed twice with pyrogen-free saline and resuspended in the same diluent to the desired concentration. All suspensions were prepared immediately before use and plate counts made before and after inoculation.

Dialysis sacs (capacity 1.5 ml) were prepared in accordance with the description given by Gladstone and Glencross except that vinyl tubing was used for the sample tubes. The sacs were implanted into the peritoneal cavity of 30 g white Swiss mice under Nembutal anesthesia; the sample tube being led out through the flank and held in place across the back with a Michel clip. After filling with saline, the sample tubes were heat-sealed, and the sac contents left to equilibrate overnight. Each mouse was fitted with a small cardboard collar to prevent it from reaching the sample tube. After equilibration, the sample tube was clamped with a hemostat (the tips covered with rubber tubing) and the sealed end aseptically cut off. The inoculum was introduced with a needle and syringe, then the entire contents mixed by repeated withdrawal and reintroduction. The total volume within the sac was noted, a small sample (approximately 0.1 ml) removed, and the sample tube resealed. The total volume within the sacs was recorded each time samples were taken.

Both plate counts and alpha toxin titrations were performed with the aid of the microtitrator (Cook Laboratories). The initial dilution for plate counts was made by charging a sterile microtitrator loop (0.025 ml) with sample and adding it to 5 ml saline. Subsequent dilutions were made in the conventional manner. Alpha toxin titrations were

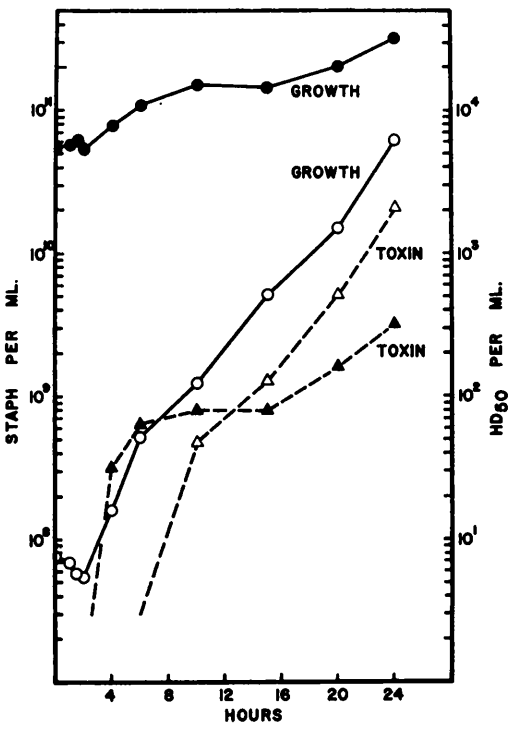

FIG. 1. Growth and alpha toxin production by *S. aureus* 18Z within dialysis sacs implanted in peritoneal cavity of mice. Open circles and triangles represent corresponding data from one experiment; solid points are those from another.

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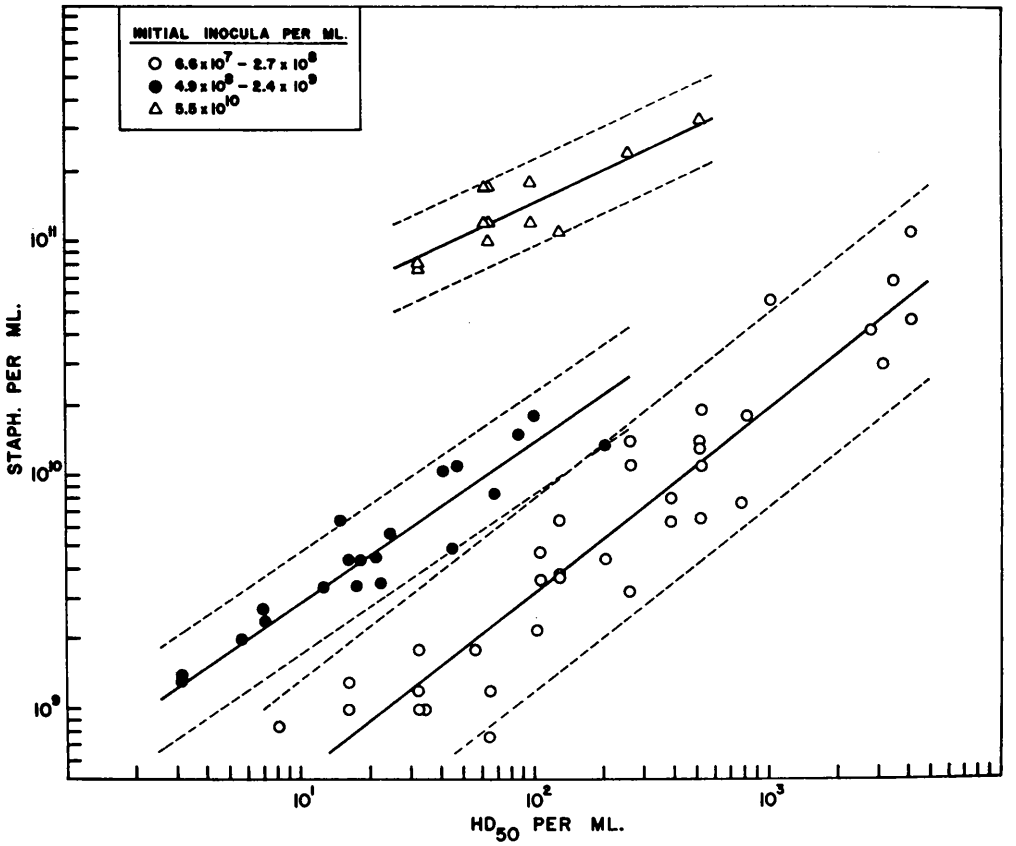

FIG. 2. Alpha toxin concentration *vs* staphylococcal concentration within dialysis sacs implanted intraperitoneally in mice. Cumulative data grouped according to size of inoculum. Solid lines are regression lines; dashed lines are respective 95% confidence limits.

done in duplicate by making serial 2-fold dilutions with the microtitrator and adding an equal volume (0.025 ml) of a 2% rabbit red blood cell suspension. Incubation was carried out at 37°C for one hour. The amount of toxin causing 50% lysis under these conditions was considered one unit (HD_{50}). Suitable corrections were made for any volume changes within the sacs throughout the experiment.

Results. It was found that *S. aureus* 18Z readily multiplied and produced alpha toxin, but it became evident that toxin production occurred only during periods of actual multiplication. In Fig. 1 are illustrated data from 2 experiments. In the one instance, when a comparatively low inoculum was used (10^8) there was a lag phase lasting about 2 hours followed by a period of multiplication which persisted for about 22 hours. Toxin production (once it became measurable) was found to parallel growth. In the other case illustrated (initial inoculum 5×10^{10}), there again was a 2 hour lag followed by a period of multiplication during which the population doubled. This was followed by another lag which lasted 4-6 hours before the population again doubled. It was evident that alpha toxin was produced only during the intervals when multiplication occurred.

By analysis of data derived from a number of experiments it was found that the concentration of alpha toxin within the sacs was not necessarily correlated with the concentration of organisms (Fig. 2). Although the concentration of toxin was usually proportional to growth within any one experiment, this relationship varied considerably with different experiments. In part this appeared due to differences in growth rates

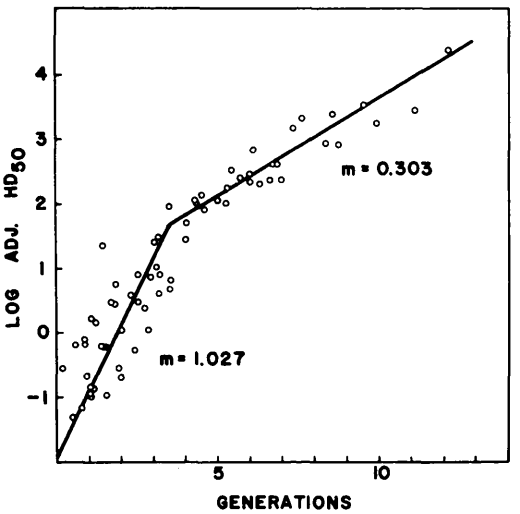

FIG. 3. Alpha toxin production by *S. aureus* 18Z with successive generations after introduction into implanted dialysis sacs. All toxin values have been adjusted to correspond to an initial staphylococcal population of 10^8 cocci/ml.

which depended on the size of the inoculum. The data in Fig. 2 have been arbitrarily grouped according to the initial inocula used in various experiments. It can be seen that the amount of toxin produced by a given number of organisms can vary significantly depending on the initial concentration of cocci within the sacs.

In view of the variations in growth rate, as well as the observation that toxin was produced only during actual multiplication, the same data were reanalyzed with respect to the amount of toxin produced with successive generations. When this was done it was found that all the data merged; however, it was obvious that the amount of toxin produced per generation was not uniform (Fig. 3). During the first 3 generations the amount of toxin produced per generation increased progressively, but after the fourth generation it became constant. In the data as presented in Fig. 3, if toxin production is constant per generation, the amount of toxin should double with every successive generation. Thus the slope of the relationship should be 0.301 (the log of 2). It is apparent that after the fourth generation our data closely approach the theoretical value. However, during the first 3 generations the rate of toxin production is much greater. This has been interpreted as being indicative of the adaptive nature of alpha toxin production under these experimental conditions.

TABLE I. Calculated Number of Alpha Toxin Molecules Produced per Coccus During Successive Generations of *in vivo* Growth. Figures in parentheses represent 95% confidence limits as derived from the experimental values. Beginning with the fifth generation toxin production per coccus per generation is maximal and remains constant thereafter.

Generation	Molecules toxin per coccus
0*	
1	850 (32- 2,200)
2	4,400 (180-12,000)
3	24,000 (950-62,000)
4	28,500 (5,200-75,000)
5	29,000 (11,000-75,000)

*160 molecules toxin associated with *in vitro* grown cell (6-400).

After these studies were completed, Bernheimer and Schwartz(3) published data concerned with their purified alpha toxin which permitted us to calculate the approximate number of toxin molecules produced per coccus with each successive generation (Table I). Although these estimates are subject to considerable error, it is at least possible to determine the order of magnitude involved.

Discussion. It is not now possible to assess the importance of these findings on the pathogenesis of staphylococcal infections. Further work is necessary to determine whether the ability to accelerate toxin production during the first few generations is important in initiation and establishment of a progressive lesion. Certainly, serious consideration is warranted concerning the manner in which staphylococci are grown for virulence studies.

Summary. When *S. aureus* 18Z grown within dialysis sacs was implanted in the peritoneal cavity of mice, alpha toxin was produced only during periods of multiplication. The amount of toxin produced per coccus increased markedly during the first 3 generations, was maximal by the fifth, and remained constant thereafter.

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2. Kapral, F. A., Shayegani, M. G., J. Exp. Med., 1959, v110, 123.
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Effect of Short Term Alpha Irradiation on Parathyroid Activity and Osteoclast Numbers. (30103)

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It is now generally accepted that the end result of the action of the classical hormone of the parathyroids is to aid in the maintenance of constant ionic calcium levels of extracellular fluids. This condition is achieved primarily through the action of the hormone on both bone and kidney. A morphological indication of increased hormonal activity, particularly in the metaphyseal region of bone, is an increase in numbers of osteoclasts. This relationship between parathyroid function and osteoclast proliferation has been recognized for many years(1-3). Also, in earlier reports, the increase in number of these cells has been studied under standardized conditions, so that the osteoclast numbers in the metaphysis of the femur of the rat could be used as an index of endogenous parathyroid activity(4,15). However, the role of the osteoclast is still not completely understood, although there is evidence to support the suggestion that it is capable of actively reabsorbing bone(5-7). Whether the cell initiates the reabsorption or phagocytizes the remnants after bone resorption has been induced by other agents, has still not been clarified. This phagocytic action of the osteoclast has been utilized by Arnold and Jee to study incorporation of plutonium into these cells(8).

The purpose of the study reported here was to attempt to destroy osteoclast function through the radiation emitted by plutonium incorporated by phagocytosis. Under such conditions it was hoped to determine whether parathyroid hormone acted through osteoclasts in its control of calcium homeostasis. In previous studies(10,11) it had been shown that the technique of peritoneal lavage in the rat stimulated endogenous parathyroid activity which caused a constant but elevated calcium and hydroxyproline transfer from bone to extracellular fluid and a proliferation in osteoclasts. Therefore, at specific times after administration of the plutonium, the lavage technique was employed to increase endogenous hormone production so that the effect of plutonium on osteoclast numbers and on bone breakdown could be studied.

Materials and methods. All experiments were conducted at the Argonne National Laboratories. The plutonium was furnished in the form of $Pu(NO_3)_4$ in 1% sodium citrate at pH 6.5. Experimental animals were male Sprague-Dawley rats, 180-200 g, maintained on a calcium-free diet for 3 days prior to use. One microcurie of plutonium was injected intraperitoneally at *one* of the following times: (1) 5 days before peritoneal lavage, (2) 24 hours before experimental use, and (3) injections of one microcurie each, at 48 hours and 24 hours before use.

The experimental procedure consisted of 8 or 12 hours of continuous peritoneal lavage, using a calcium-free, phosphate-free buffered rinse. This method has been explained in detail(12). Calcium in the sera and in the hourly lavage samples was analyzed using a flame photometric method(13). The analysis for free hydroxyproline in the sera and lavage washes has been previously described(14).

Histology. Immediately after the lavage procedure, the rats were sacrificed and both femora and in some cases, mandibles, vertebrae and liver tissues were removed and fixed

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