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## Studies of Staphylococcal Infections. I. Virulence of Staphylococci and Characteristics of Infections in Embryonated Eggs\*

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Many of the determinants of the pathogenesis and course of staphylococcal infections remain imprecisely defined (1, 2) despite their increasing importance (3-10). Experimental infections in suitable laboratory animals have been of considerable assistance in clarifying the role of host defense mechanisms and specific bacterial virulence factors with a variety of other infectious agents. A sensitive experimental model would be of value in defining the importance of these factors in staphylococcal infections, but both humans and the usual laboratory animals are relatively resistant. Extremely large numbers of staphylococci are required to produce either local or systemic infections experimentally. Elek was unable to produce local purulent lesions in humans with inocula of less than  $10^8$  staphylococci (11) but subsequently demonstrated that the infectious inoculum could be reduced by two to four logs when the infection was combined with concomitant insertion of a foreign body (12). Even larger numbers of staphylococci have been required to produce local lesions in mice (13, 14), guinea pigs (15, 16), and rabbits (17, 18). Various traumatic procedures or implantation of foreign bodies also allows the production of infection with smaller numbers of bacteria in animals (14, 19), but despite such measures many of these tech-

niques still require relatively large numbers of staphylococci to produce infection (19). Fatal systemic infections have been equally difficult to produce in animals and have necessitated the injection of  $10^7$  to  $10^9$  bacteria (20-23). A few strains of staphylococci have been found that are capable of producing lethal systemic infections with inocula of from  $10^2$  to  $10^3$  bacteria (24) and have excited considerable interest (25-27). The virulence of these strains apparently results from an unusual antigenic variation (27, 28) which, despite its interest, is of doubtful significance in human staphylococcal infection, since such strains have been isolated only rarely from clinical infections (29). The insensitivity of these experimental systems has been a handicap for comparison of the relative virulence of strains of staphylococci and evaluation of the effect of antibody and other defense mechanisms in a living system.

Preliminary studies of staphylococcal infections in a variety of animals indicated that embryonated eggs were readily infected with pathogenic staphylococci (30). The present study describes the sensitivity of embryonated eggs to 68 strains of coagulase positive *Staphylococcus aureus* and 36 strains of coagulase negative *Staphylococcus epidermidis* and defines some of the characteristics of this infection. Subsequent publications will describe the importance of individual bacterial virulence factors (31) and the protective effect of antitoxic and antibacterial antibody in the chick embryo (32).

### Methods

*Strains of staphylococci.* Forty-two strains of coagulase positive *Staphylococcus aureus* were obtained from blood cultures and specimens of sputum or purulent exudates submitted to the routine bacteriology laboratory. In each instance, the patient from whom the

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specimen was obtained was examined to insure that the staphylococcus isolated was etiologically related to the clinical infection. Additional strains were obtained from the anterior nares and skin of a group of patients without clinical evidence of infection who were attending an outpatient clinic for the first time. Specimens were obtained only from outpatients who had not received antibiotics within the past 3 months, had not been hospitalized during this period, and whose household contacts had not been hospitalized recently. A third group of 36 strains of coagulase negative *Staphylococcus epidermidis* was obtained from a variety of specimens submitted to the hospital laboratory and from the nares and skin of outpatients.

Tube and slide coagulase determinations and mannitol fermentation were performed on all isolated strains to insure their identification. Strains of staphylococci were maintained on tryptose phosphate (TP) agar slants at 0° C after isolation and identification until egg virulence studies were performed the following week. Immediately before use, a single colony selected from a subculture of the original agar slant was inoculated into TP broth for the infectious inoculum. Several 0.1-ml samples of the latter were transferred to 2.5 ml of TP broth in screw-capped vials and stored in a -20° C deep freeze for subsequent use.

*Preparation and injection of inocula.* Serial tenfold dilutions of overnight TP broth cultures of staphylococci were made to provide inocula of approximately 1,000 to 99,999 ( $10^5$  to  $10^6$ ) and 1 to 100 colony forming units (cfu). The smaller inocula consisted of less than 10 cfu in over one-half the studies. The size of the inoculum was determined by plating 0.1 ml of  $10^{-5}$ ,  $10^{-6}$ , and  $10^{-7}$  dilutions of the overnight culture on TP agar and enumerating the number of colonies after overnight incubation. Lots of 20 10-day-old fertile eggs were injected intra-allantoically with 0.1 ml of  $10^{-3}$  and  $10^{-6}$  dilutions of each strain studied, and the eggs were candled daily to determine viability. A group of ten controls was injected with a similar volume of sterile 0.85% saline (PS) in each experiment. Subcultures were obtained from allantoic fluid, yolk, and embryonic blood in the first sets of experiments and, when indicated, in subsequent studies to insure that maternal transmission of infection or subsequent contamination had not occurred.

*Eggs.* Fertilized eggs were obtained from a commercial source that did not use an antibiotic dietary supplement. Tube dilution assays for antibacterial activity were performed on allantoic and amniotic fluid on several occasions with negative results. Eggs were incubated for 10 days at 37° C in a rocker incubator before infection and then incubated upright at 38.5° C with 60% relative humidity for an additional 10 days after infection.

*Recovery of inoculum.* Studies to determine the number of cfu that could be recovered immediately after injection were carried out by two techniques. Eggs were injected as described above, allowed to stand for 15 minutes, sealed with paraffin, shaken gently, and sacrificed.

When recoveries were carried out after injection of a large inoculum, 12-day-old eggs in which the volume of allantoic fluid is stated to be 6 ml (33, 34) were used for injection. One-tenth ml samples of allantoic fluid were aspirated aseptically after removal of the shell over the egg sac, plated, the colonies enumerated, and the numbers corrected for the 1 to 60 dilution. Recovery with the smaller inoculum was performed similarly, except as much of the allantoic fluid as possible was aspirated and 4 ml of PS was injected, mixed, and removed. The latter procedure was repeated nine times, and the pooled allantoic fluid and saline washings were filtered through a Millipore H.A. filter (0.45  $\mu$ ). After filtration, the filter was removed, placed on a blood agar plate, incubated for 48 hours, and the number of bacterial colonies determined.

*In vitro growth curves.* Allantoic fluid was collected aseptically from 15 eggs, pooled, and 4.9 ml dispensed into sterile capped tubes for determination of growth curves. One-tenth ml of a  $10^{-6}$  dilution of the organism to be tested was added and the allantoic fluid incubated for 18 hours. Duplicate determinations were made at each sampling time. Concomitant growth curves in TP broth were performed in a similar fashion.

*In vivo growth curves.* Lots of 64 10-day-old embryonated eggs were injected with 0.1 ml of a  $10^{-3}$  and  $10^{-6}$  dilution of coagulase positive staphylococci and 0.1 ml of a  $10^{-3}$  dilution of coagulase negative staphylococci. Four eggs from each of the three groups were sacrificed at 4, 8, 18, and 24 hours and daily thereafter for 10 days. Only eggs containing living embryos were sacrificed to insure that acceleration of growth and invasion of the embryo "post-mortem" did not obscure the results. The shell over the air sac was removed; allantoic fluid was aspirated aseptically and serially diluted for plate counts. The remainder of the allantoic fluid was removed, and 1.5% saponified cresylic acid<sup>1</sup> was added to the shell containing the intact yolk sac, amnion, and embryo. After 10 minutes, the cresylic acid solution and the yolk were removed. The amnion and embryo were extracted with sterile forceps and placed in a sterile petri dish. The amnion was removed and the embryo washed in the cresylic acid solution and rinsed with sterile PS. The embryo was opened by a ventral incision, and the heart and liver were removed aseptically. These were ground in a glass tube with a Teflon tissue grinder, and 0.2 ml of the bloody supernatant fluid was removed. Bacterial titers were determined by plating 0.1 ml of undiluted fluid and serial tenfold dilutions of the fluid.

## Results

*Lethality of coagulase positive and coagulase negative staphylococci.* The 10-day fatality rates for 10-day-old eggs injected intra-allantoically with inocula of two different sizes of 68 strains of co-

<sup>1</sup> Cresylone, Parke, Davis & Co., Detroit, Mich.

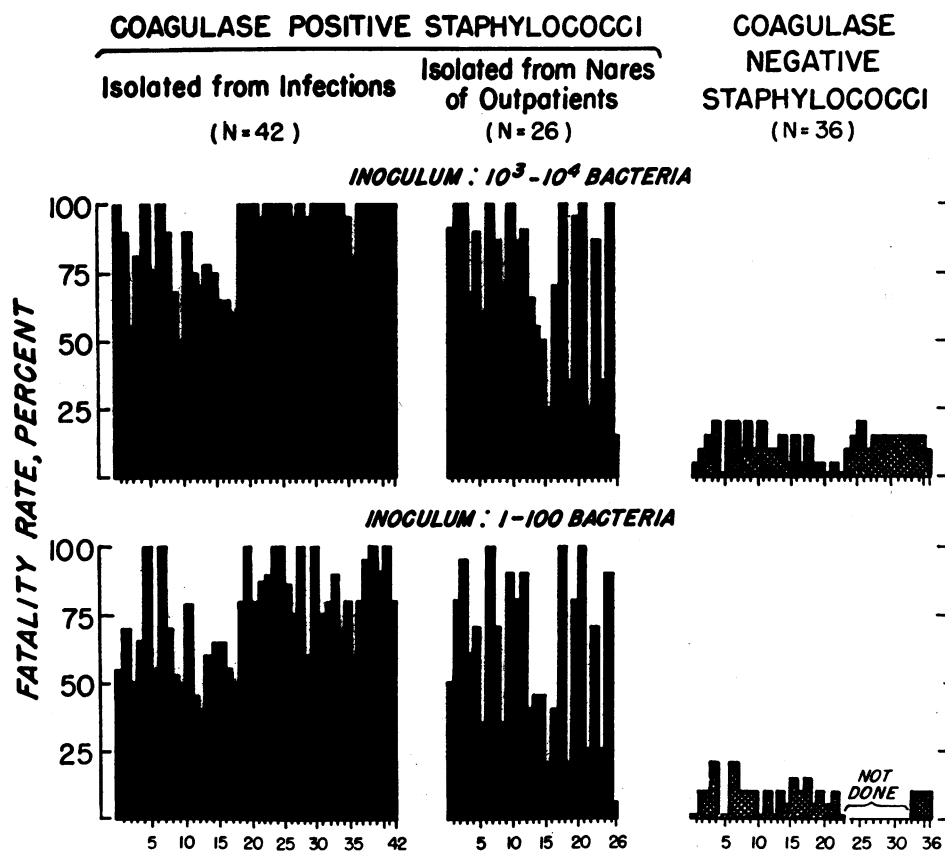


FIG. 1. CHICK EMBRYO LETHALITY OF COAGULASE POSITIVE STAPHYLOCOCCI ISOLATED FROM INFECTIONS COMPARED WITH THAT OF STRAINS ISOLATED FROM NASAL CARRIERS AND COAGULASE NEGATIVE STAPHYLOCOCCI.

agulase positive *Staphylococcus aureus* and 36 strains of coagulase negative *Staphylococcus epidermidis* are illustrated in Figure 1. Two groups of coagulase positive staphylococci were studied. Forty-two strains were isolated from unequivocal human infections in which they were the sole etiologic agent of the infections. These strains are referred to, henceforth, as pathogenic staphylococci. A second group of 26 strains isolated from the nares and skin of uninfected outpatients represents staphylococci of uncertain pathogenicity. The latter group, whose over-all human pathogenicity, although uncertain, would be anticipated to be less than that of strains isolated from definite human infections, was selected to sample strains characteristic of those prevalent in the community. The third group, coagulase negative *Staphylococcus epidermidis*, represented strains that are rarely human pathogens.

Pathogenic staphylococci produced fatality rates ranging from 50 to 100%, mean  $89.2 \pm 14.9$ , when  $10^8$  or  $10^4$  cfu was injected, and from 40 to 100%, mean  $75.8 \pm 18.5$ , with inocula of less than 100 cfu. Fatality rates of 50% or greater occurred with all strains isolated from human infections with the larger inocula, and with 40 of 42 of these strains with the smaller inocula. The lethality of the 26 strains of coagulase positive staphylococci isolated from outpatients was between 15 and 100%, mean  $72.8 \pm 26.9$ , with the large inocula and from 5 to 100%, mean  $60.0 \pm 29.3$ , with the small inocula. Both the large and small inocula of pathogenic strains were significantly more lethal ( $t_{66} = 4.5$ ,  $p < 0.001$ ;  $t_{66} = 3.95$ ,  $p < 0.001$ ) than were equivalent inocula of strains isolated from outpatients. Although strains that had produced clinical infections were more virulent for embryonated eggs in general than

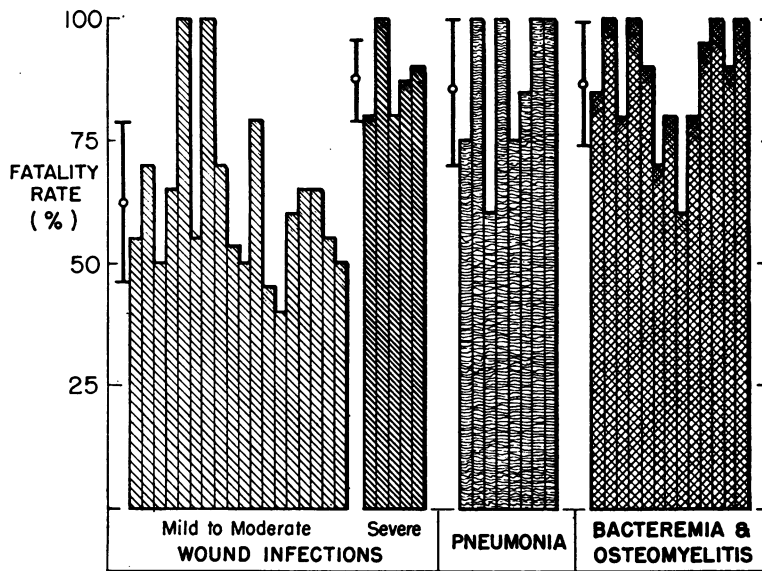


FIG. 2. LETHALITY OF STRAINS OF STAPHYLOCOCCUS AUREUS ISOLATED FROM INFECTIONS OF VARIOUS TYPES AND DEGREES OF SEVERITY.

strains isolated from uninfected patients, considerable overlap in egg virulence was observed with individual strains of both types.

Coagulase negative strains were significantly less lethal than coagulase positive staphylococci isolated either from septic patients or uninfected outpatients. Fatality rates did not exceed 20% either with inocula of 1 to 100 or  $10^3$  to  $10^4$  cfu, and the mean fatality rate with the larger inocula,  $11.0 \pm 6.1\%$ , did not differ significantly ( $t_{66} = 1.65$ ,  $p = 0.1$ ) from the fatality rate with the smaller inocula,  $8.3 \pm 5.8\%$ . The mean fatality rate produced by 1 to 100 cfu of coagulase positive staphylococci isolated from outpatients was significantly greater ( $t_{66} = 10.1$ ,  $p < 0.001$ ) than that produced by 100 to 1,000 times as many coagulase negative staphylococci.

*Lethality of strains isolated from infections of varying severity.* Figure 2 demonstrates the fatality rates observed in embryonated eggs after injection of 1 to 100 cfu of strains of pathogenic staphylococci isolated from different types of clinical infections. Strains isolated from patients with staphylococcal bacteremia, pneumonia, and wound infections were studied. Five patients had particularly extensive and severe wound infections, and the strains isolated from these patients are distinguished from strains causing less severe infections in Figure 2. Severe wound infections

were differentiated by the following criteria: occurrence in patients without serious underlying disease, extensive inflammatory reaction, leukocytosis greater than 17,000 per  $\text{mm}^3$ , and fever in excess of  $102^\circ \text{F}$  for 5 days or longer. All other wound infections were classified as being of mild to moderate severity when two or fewer of the above criteria were present. The latter infections usually consisted of stitch abscesses and infections producing moderate purulent drainage and wound induration with only slight systemic symptoms. No further attempt was made to classify the severity of infections except in these five instances. Staphylococcal pneumonia was associated with bacteremia in two patients; these strains are shown in both categories. Three patients with primary hematogenous osteomyelitis were observed, and their strains are grouped with strains isolated from patients with bacteremia. The mean fatality rate from infections with strains causing mild to moderate wound infections was  $62.6 \pm 17.0\%$  and was significantly less ( $t_{21} = 3.5$ ,  $p < 0.01$ ;  $t_{26} = 3.6$ ,  $p < 0.01$ ;  $t_{28} = 4.7$ ,  $p < 0.001$ ) than the fatality rate of infections with strains isolated either from patients with severe wound infections (87%), pneumonia (86%), or bacteremia (87%). Even when strains associated with severe wound infections were combined with those that produced mild to mod-

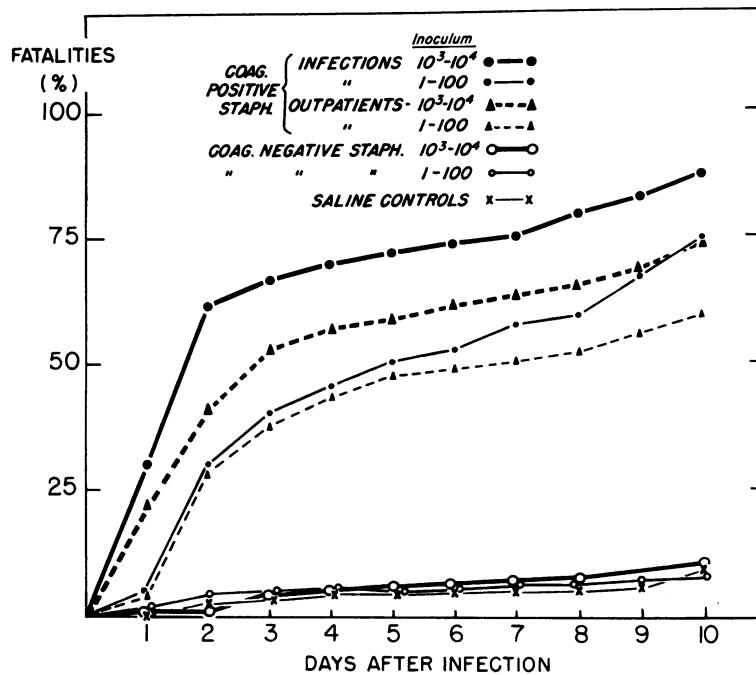


FIG. 3. CUMULATIVE FATALITY RATES AFTER ALLANTOIC INFECTION WITH STRAINS OF STAPHYLOCOCCUS AUREUS AND STAPHYLOCOCCUS EPIDERMIDIS.

erate infections, their over-all lethality was significantly less than that of strains isolated from pneumonia and bacteremia. Lethality did not differ materially, however, for strains isolated from patients with severe wound infections, pneumonia, or bacteremia.

*Size of inoculum and rapidity of death.* The increase in fatality attained with a 1,000-fold increase in inoculum size over that observed with a small inoculum often was not so great as might have been anticipated. This finding appeared to reflect resistance of a small proportion of the eggs to fatal infection in addition to differences in the rate of killing by inocula of 100 and  $10^3$  to  $10^4$  staphylococci during the period of observation. The effect of the latter may be seen in part in the cumulative daily death rates with the two sizes of inocula of the three groups of staphylococci illustrated in Figure 3. Larger inocula of pathogenic staphylococci were more rapidly fatal and produced 70% of the total fatalities within the first 2 days after infection, and only a small proportion of deaths occurred later in the 10-day period. With smaller inocula, there was an initial lag before deaths were apparent, and the death

rate increased progressively until the final day of observation, with 60% of the total fatalities occurring after the second day. A similar but less striking relation between the size of the inoculum and rapidity of fatalities also occurred with strains isolated from uninfected outpatients. The effect of the size of the inoculum on the time of death was even more apparent when individual strains were considered. More virulent strains that produced fatality rates of 85% or greater with a small inocula produced 84% of their fatalities in the first 2 days after infection with  $10^3$  bacteria, whereas less lethal strains produced only 47% of their fatalities within this time ( $\chi^2 = 7.3$ ,  $p < 0.01$ ).

*Lethality of coagulase negative staphylococci and culture media.* Since coagulase negative staphylococci produced very few fatalities in the comparative studies, their virulence was assessed more critically by comparing the lethality of five strains with that of the culture media. Tenfold dilutions of overnight TP broth cultures were made in sterile PS, and 0.1 ml of undiluted cultures and  $10^{-1}$ ,  $10^{-2}$ ,  $10^{-3}$  dilutions were injected intra-allantoically into ten eggs per dilution with

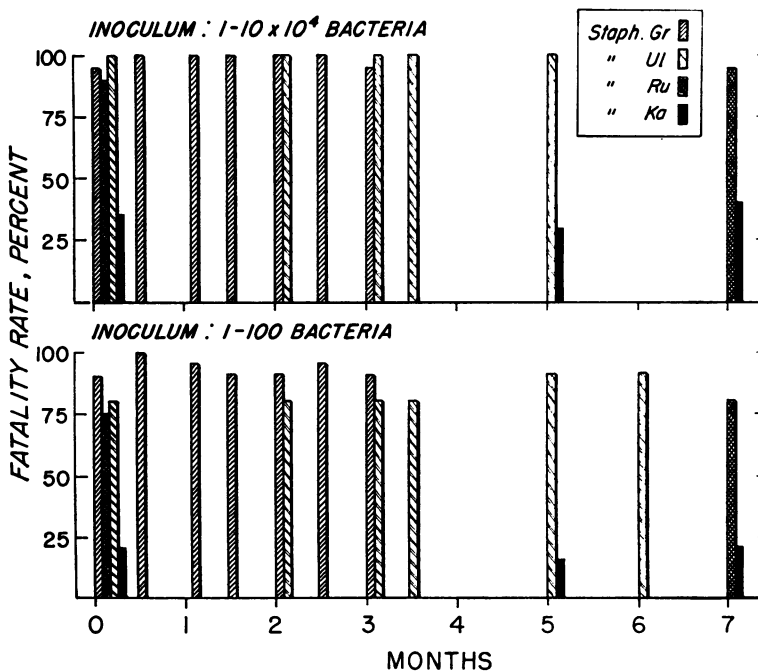


FIG. 4. REPRODUCIBILITY OF FATALITY RATES IN EMBRYONATED EGGS OF FOUR STRAINS OF STAPHYLOCOCCUS AUREUS STUDIED OVER A 7-MONTH PERIOD.

particular care being taken to minimize trauma to the eggs. Sterile TP broth was diluted and injected in a similar fashion, and the number of deaths was determined daily for 10 days for all five groups. The inoculum varied from  $1 \times 10^7$  to  $2 \times 10^8$  cfu with undiluted cultures. The number of survivors in each group is shown in Table I. No significant differences were observed among any of the groups, and undiluted overnight cultures of coagulase negative staphylococci were no more lethal than the media in which they were grown.

*Recovery of injected staphylococci.* Some leakage occurred after the injection of bacteria in

many instances. To insure that a large proportion of the injected organisms was not lost, recoveries were carried out with two different sizes of inoculum as described previously. The results of four such recoveries are shown in Table II. In each instance, recoveries approximated 60% of the bacteria injected and were well within the range of error of the experimental method.

*Reproducibility of fatality rates.* The virulence of individual strains was reproducible over a period of several months. Serial assessments of the lethality of three virulent strains and one nonvirulent strain, shown in Figure 4, were made at intervals of as long as 7 months. Fatality

TABLE I  
Comparative lethality of coagulase negative staphylococci and sterile culture media

Dilution	Sterile broth	Strains of coagulase negative staphylococci				
		F	R	B	K	S
Undiluted	9/10*	9/10	8/10	10/10	9/10	8/10
10 <sup>-1</sup>	10/10	10/10	10/10	9/10	10/10	10/10
10 <sup>-2</sup>	9/10	10/10	10/10	10/10	10/10	9/10
10 <sup>-3</sup>	10/10	10/10	9/10	9/10	10/10	10/10

\* No. of survivors at 10 days/no. of eggs injected.

TABLE II  
Recovery of injected bacteria from allantoic fluid

No. of bacteria		Per cent recovered
Injected	Recovered	
cfu*	cfu	%
$3.1 \times 10^4$	$1.8 \times 10^4$	58
$4.5 \times 10^4$	$3.0 \times 10^4$	67
7	4	59
7	4	59

\* cfu = colony forming units.

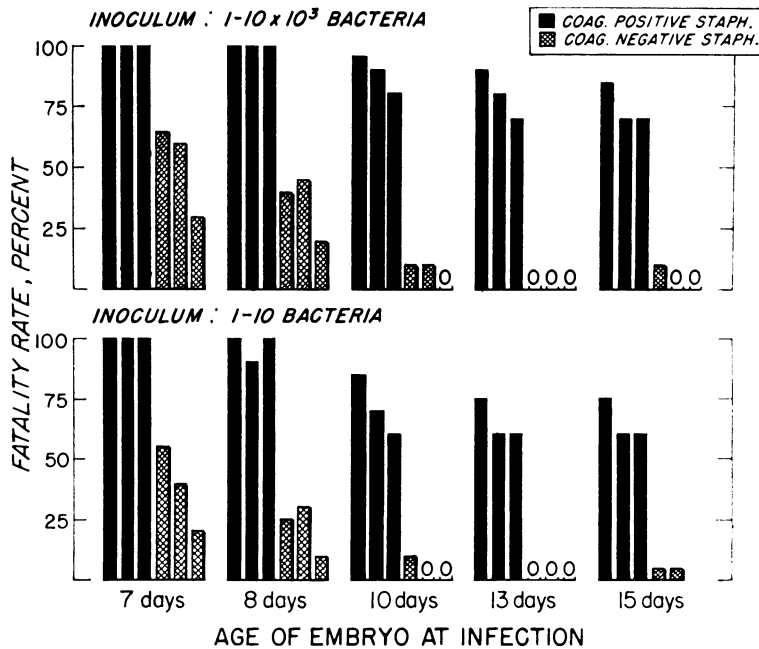


FIG. 5. SUSCEPTIBILITY OF EMBRYONATED EGGS OF VARIOUS AGES TO LETHAL INFECTION WITH *STAPHYLOCOCCUS AUREUS* AND *STAPHYLOCOCCUS EPIDERMIS*.

rates did not vary more than 10% even when studies were made after prolonged intervals. A number of other strains not shown in this Figure have been studied on several occasions with equally reproducible results.

*Susceptibility of embryos of different ages.* The age of the embryo exerted a significant influence on its susceptibility to lethal infection with both coagulase positive and negative staphylococci. The five-day fatality rates produced by three strains of coagulase positive and by three strains of coagulase negative staphylococci for 7-, 8-, 10-, 13-, and 15-day-old chick embryos are shown in Figure 5. Seven- and, to a lesser degree, 8-day-old embryos were susceptible to lethal infections with coagulase negative strains but became resistant by the tenth day. The age of the embryo modified the virulence of coagulase positive strains in a similar fashion. Almost uniform fatality occurred in 7- or 8-day old embryos; 13- and 15-day-old embryos were less susceptible. The greater susceptibility of the younger embryos did not obscure the differences in virulence between coagulase positive and coagulase negative strains. The former strains were significantly more viru-

lent at all ages, although the differences were most evident in embryos 10 days of age or older.

*Growth of staphylococci in allantoic fluid in vitro.* Growth curves in allantoic fluid *in vitro* were determined for three strains of coagulase negative staphylococci and three strains of coagulase positive staphylococci and are compared with growth curves of each in TP broth in Figure 6. Multiplication of coagulase negative staphylococci in allantoic fluid lagged behind that observed in broth and was less than that of coagulase positive strains in either type of medium during the first 6 hours. This difference failed to persist, however, and both types attained similar population densities of  $10^8$  bacteria per ml by 18 hours in both media.

*Growth of staphylococci in vivo.* *In vivo* growth curves also were determined in both allantoic fluid and the embryo after injection of both a large ( $2 \times 10^8$  cfu) and small (2 cfu) inoculum of coagulase positive staphylococci, and a large inoculum ( $9 \times 10^8$  cfu) of coagulase negative staphylococci. These growth curves plus the cumulative daily fatality rates of the strains studied are illustrated in Figure 7. Points on the graph



represent the mean of counts obtained from four eggs in each of three experiments. Growth curves of the large inoculum of coagulase positive and coagulase negative staphylococci were quite similar for the first 3 days with both reaching numbers of  $10^8$  cfu per ml or greater in allantoic fluid and  $10^3$  to  $10^4$  cfu in the embryo. The high early fatality rate in eggs injected with  $10^3$  cfu of pathogenic staphylococci precluded assessment of growth beyond this period, although this measurement was attempted with lots of 64 eggs on three separate occasions. After the injection of a small number of coagulase positive staphylococci (2 cfu), 48 hours elapsed before their numbers reached those attained by the larger inoculum, but after this period the microbial population persisted essentially unchanged in both allantoic fluid ( $10^8$  per ml) and the embryo ( $10^3$  to  $10^4$ ) for the remainder of the study. In contrast, after an initial rise, a progressive decrease in the number of coagulase negative staphylococci was observed in both the embryo and allantoic fluid until the number of coagulase negative staphylococci present on day 3 had decreased by 99.7% in the allantoic fluid, and by 99.2% in the embryo by the tenth day. All three experiments gave similar results, with coagulase positive staphylococci attaining similar numbers in 48 hours and persisting at this level, while the

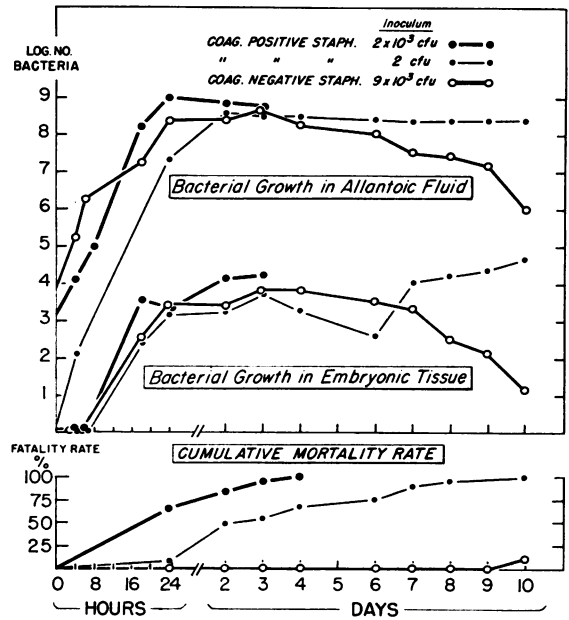


FIG. 7. GROWTH CURVES AND CUMULATIVE FATALITY RATES OF STAPHYLOCOCCUS AUREUS AND STAPHYLOCOCCUS EPIDERMIDIS IN ALLANTOIC FLUID AND EMBRYONIC TISSUE IN VIVO. Each point on the growth curves represents the mean of counts obtained from four eggs in each of three experiments.

progressive decrease in numbers of coagulase negative staphylococci after days 3 and 4 also occurred in each study.

Invasion of the embryo was observed only when bacterial counts in the allantoic fluid exceeded  $10^7$  cfu per ml. Involvement of the embryo appeared to be related to increasing fatalities with coagulase positive staphylococci, but coagulase negative staphylococci were capable also of invading the embryo without producing fatalities.

Discussion

These investigations were undertaken in an attempt to find a more sensitive experimental host for the study of staphylococcal infections. Embryonated eggs seem to possess many features that would be desirable in such an experimental model. They are extremely susceptible to infection with small numbers of pathogenic staphylococci, and the lethality of individual strains is consistent and reproducible over a period of several months. The sensitivity to infection also may be modified by utilizing the differences in susceptibility of em-

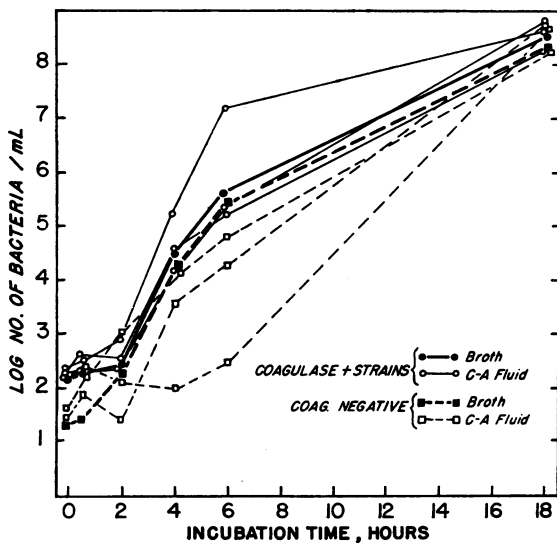


FIG. 6. GROWTH CURVES OF STAPHYLOCOCCUS AUREUS AND STAPHYLOCOCCUS EPIDERMIDIS IN ALLANTOIC FLUID AND BROTH MEDIA.

bryos of various ages if greater or lesser lethality is desired. In addition, a distinct relationship between the pathogenicity of staphylococci for humans and their virulence for chick embryos was observed, confirming earlier preliminary findings (30). Strains of *Staphylococcus aureus* definitely responsible for human infections were extremely lethal and almost uniformly produced fatality rates of 50% or greater when less than 100, and usually less than 10 cfu, were injected. Other strains of indeterminate potential pathogenicity to man, isolated from uninfected carriers, exhibited, individually, variable degrees of virulence for the chick embryo but as a group were significantly less lethal than strains isolated from clinical infection. Fatality rates from infection with strains that are avirulent or noninvasive for humans, coagulase negative *Staphylococcus epidermidis*, were no greater than those occurring after injection of culture media similar to that used for their propagation. Further correlation between the severity of the clinical infections and the virulence of individual strains for chick embryos was also observed. Staphylococci isolated from patients with bacteremia or pneumonia were more lethal than strains isolated from wound infections, and strains isolated from more severe wound infections produced greater fatality rates than strains isolated from less severe wound infections.

The excellent correlation observed between the pathogenicity and virulence of staphylococci for humans and their lethality in chick embryos does not imply, however, that this system can be utilized to predict the pathogenicity of staphylococci for humans. The parameters used to characterize clinical behavior of staphylococci are relatively imprecise, and the potential pathogenicity of individual staphylococci cannot be predicted accurately by any currently available techniques. Precise quantification and assignment of relative degrees of virulence will require thorough epidemiologic investigation of the frequency and types of human infection in relation to contact and acquisition of individual strains as well as concomitant appraisal of predisposing host factors. Until such information is available, definitive correlation between the similarity of behavior of staphylococci in humans and animals will not be possible.

Embryonated eggs have been used predominantly for the propagation and study of viruses and rickettsiae, although Goodpasture recognized their potential value for investigation of bacterial infections (35) soon after he popularized their use for cultivation of viruses (36). One of his early studies with this model described the lesions produced by infection of allantoic membranes and the ability of a variety of bacteria, including staphylococci, to invade the membrane and produce lethal infections (35). Subsequently, Frappier and Sonea observed that staphylococci injected subcutaneously into 18- or 19-day-old embryos produced lethal infections (37), and Knothe studied a variety of allantoic infections including those produced by staphylococci (38). The extreme sensitivity of chick embryos to staphylococci was noted in both of these investigations and more recently by Wiley, who determined the lethality of four strains of *Staphylococcus aureus* in embryonated eggs (39). However, the limited number of strains evaluated and the failure to further extend these observations has hampered recognition of the potential value of this model.

Explanation of the marked differences in virulence of coagulase positive and coagulase negative staphylococci for embryonated eggs is not readily apparent from these studies. Both types of organisms grew equally well in allantoic fluid *in vitro* and initially *in vivo*, and both invaded the embryo with equal facility, but subsequently their behavior differed. Pathogenic staphylococci persisted at essentially the same microbial population attained after logarithmic growth in both allantoic fluid and the embryo throughout the entire infection. In contrast, although coagulase negative staphylococci reached a population similar in magnitude to that of pathogenic staphylococci, they were nonlethal, and a progressive decrease in their numbers occurred after the third day until only 0.3% of the maximal population attained in the allantoic fluid and only 0.8% attained in the embryo remained by the tenth day after infection. A variety of enzymes and exotoxins has been purported to be responsible for the virulence of coagulase positive staphylococci and may play an important role in infections caused by these organisms in the chick embryo. If exotoxins do exert a significant influence on

the course of the infection, production of exotoxin *in vivo* rather than injection of preformed exotoxin is responsible. Previous studies have demonstrated that the lethality after injection of washed and unwashed staphylococci does not differ materially and that heat-killed cultures are nontoxic to the embryo (30). Alpha hemolysin is often listed as being one of the more important of the toxins elaborated by staphylococci because of its hemolytic, dermonecrotic, leukocytic, and lethal properties. Only negligible amounts of  $\alpha$ -hemolysin are produced by the cultural techniques utilized for the preparation of the infectious inoculum, but production *in vivo* does occur in infected chorioallantoic fluid (31, 40). Recent investigations by Goshi, Cluff, and Norman have suggested that the leukocytic action of  $\alpha$ -hemolysin produced *in vivo* inhibits phagocytosis and allows enhanced proliferation of coagulase positive staphylococci in infections in rabbits (41). A similar effect of  $\alpha$ -hemolysin may have been the mechanism allowing persistence of large numbers of coagulase positive staphylococci in the embryo at a time when the microbial population of coagulase negative staphylococci was progressively decreasing. Production of  $\alpha$ -hemolysin does not entirely explain the lethality of staphylococci in this system, however. Studies, yet to be reported, have demonstrated that antitoxin does not significantly alter the lethality of the infection although it completely inactivates  $\alpha$ -hemolysin produced *in vivo* (32). In addition, strains of staphylococci, lethal to embryonated eggs, have been studied which fail to produce  $\alpha$ -hemolysin either *in vivo* or *in vitro* (31). A similar situation is found in human infections where  $\alpha$ -hemolysin, although produced by most staphylococci isolated from human infections, is not a prerequisite for human pathogenicity (42, 43). Other exotoxins may be equally or more important than  $\alpha$ -hemolysin in this experimental system, and attempts are in progress to delineate their role and the exact cause of death of the embryo.

### Summary

Studies with embryonated eggs demonstrated that staphylococci pathogenic for man are extremely lethal to this host. Intra-allantoic infection with less than 100 colony forming units of

strains of *Staphylococcus aureus* isolated from clinical infections almost uniformly produced fatality rates of 50% or greater. The virulence of individual strains was reproducible over prolonged periods and tended to parallel their clinical behavior. Staphylococci isolated from clinical infections were more lethal than strains obtained from healthy carriers, strains isolated from severe staphylococcal infections were more lethal than strains isolated from milder infections, and strains of *Staphylococcus epidermidis* were essentially nonlethal for chick embryos. The age of the embryo also modified lethality, embryos of less than 10 days of age being more susceptible to fatal infection with both coagulase positive and coagulase negative staphylococci than older embryos.

Differences in growth between coagulase positive and coagulase negative staphylococci during infection were observed but did not explain the marked differences in virulence of the two species. Both grew equally well in allantoic fluid *in vitro* and *in vivo* and invaded the embryo. Coagulase positive staphylococci persisted at essentially the same numbers that were attained after maximal growth in allantoic fluid and in the embryo, whereas the numbers of coagulase negative staphylococci progressively decreased after initial logarithmic growth until less than 1% remained viable 10 days after infection. The mechanism of death for the embryo, the role of exotoxins, and explanation of the differences in virulence of coagulase positive and coagulase negative staphylococci remain to be determined.

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