EXPERIMENTAL INFECTION OF THE SKIN IN THE HAMSTER SIMULATING HUMAN IMPETIGO

III. Interaction Between Staphylococci and Group A Streptococci*

By ADNAN S. DAJANI,‡ M.D., and LEWIS W. WANNAMAKER,§ M.D.

(*From the Departments of Pediatrics and Microbiology, University of Minnesota Medical School, Minneapolis, Minnesota 55455*)

(Received for publication 21 May 1971)

In human impetigo, Group A beta hemolytic streptococci and *Staphylococcus aureus* are the organisms most frequently implicated as etiologic agents (1–8). The majority of *S. aureus* strains recovered in pure form from skin lesions belong to phage Type 71 (1, 2, 5–7, 9). Strains of this staphylococcal phage Type have a number of unusual biological activities. Penicillin G resistance is common among phage Type 71 staphylococci (5, 9). These organisms produce opacity in horse serum agar but not in egg yolk broth medium (9). On solid media, *Corynebacterium diphtheriae* is inhibited by these staphylococci (1, 9).

In previous work from our laboratory a bactericidal substance active in vitro against a variety of Gram-positive microorganisms has been isolated from culture supernates of phage Type 71 staphylococci (10, 11). This extracellular proteinaceous material has properties characteristic of some classes of bacteriocins (11). It inhibits protein and DNA synthesis and degrades RNA in susceptible cells (12), with concomitant changes in intracellular appearance (13).

The present communication is an extension of these in vitro studies. It documents the bactericidal effect of phage Type 71 staphylococci in vivo. The interaction between Group A streptococci and staphylococci is explored in an experimental hamster model for impetigo (14, 15) used previously in this laboratory to study the natural history of the infection (15) and the effect of various therapeutic regimens (16).

Materials and Methods

Organisms.—Two strains of Group A beta hemolytic streptococci were used. PF 1643, an M Type 57 strain, was isolated from a skin lesion of a patient who subsequently developed

* These studies were conducted under the sponsorship of the Commission on Streptococcal and Staphylococcal Diseases, Armed Forces Epidemiological Board, and supported by the U.S. Army Medical Research and Development Command under contracts No. DADA-17-70-C-0081 and No. DADA-17-70-C-0082 and in part by a grant from the Graduate School of the University of Minnesota.

‡ Supported by U.S. Public Health Service Research Career Development Award from the National Institute of Allergy and Infectious Disease.

§ Career Investigator of the American Heart Association.

acute glomerulonephritis. Strain 70-711 is M Type 12 and was isolated from a blood culture of a patient at Hennepin County General Hospital, Minneapolis, Minn. Both strains were grown in Todd-Hewitt broth (Difco Laboratories, Inc., Detroit, Mich.) for 6 hr at 37°C and 2 ml portions were then stored at −65°C until needed. Before use, specimens were thawed, reinoculated into fresh Todd-Hewitt broth, grown for 4–6 hr at 37°C, and the number of viable colony-forming units (CFU)[1] per milliliter determined.

The three *Staphylococcus aureus* strains employed were of different phage types. Strain C55, used as a prototype of phage Type 71 staphylococci in these studies, was isolated from an impetiginous lesion and has been shown to produce a bactericidal substance in vitro (10, 11). Strains RL 995 and RL 3809 are phage Types 75 and 81, respectively, and were also originally isolated from lesions of the skin. Neither of these latter two strains produces a bactericidal substance as tested by the in vitro method described previously. All staphylococcal strains were grown in tryptic soy broth (TSB) (Difco) and stored as above for the streptococcal strains.

Assay for Bactericidal Substance.—The lawn-spotting method for bactericidal activity against Group A streptococci was used as previously described (10). Supernatant fluids from 24–36 hr cultures of the staphylococcal strains in TSB were employed (10, 11).

Animal Inoculation.—The method for production of skin lesions in Syrian hamsters was reported previously (15). In brief, 0.1 ml of a 4–6 hr culture of the various organisms, or mixtures of organisms, were injected intradermally into the shaved backs of hamsters using a 27-gauge needle on a tuberculin syringe. Each animal was injected at four sites at least 3 cm apart.

Quantitation of Bacteria in Lesions.—Cultures from lesions were obtained after removal of scabs if present. A calibrated platinum loop, delivering 0.001 ml, was used to transfer material from the base of a lesion to 1.0 ml of sterile 0.85% NaCl solution. After adequate mixing, serial dilutions were made in the saline and 0.1 ml portions from various dilutions were plated immediately onto the surface of blood agar with and without crystal violet ($1:10^6$ final concentration). After incubation at 37°C for 18–24 hr, the number of viable CFU per loop was calculated.

RESULTS

To assess the effect of various strains of staphylococci on the survival of streptococci in experimental lesions, mixtures of the two organisms were injected into the skin of hamsters. A suspension of logarithmic-phase organisms of M Type 57 streptococcus containing 1.2×10^8 CFU/ml were mixed with equal volumes of the three staphylococcal strains belonging to phage Types 71, 75, and 81, respectively. All staphylococcal cultures contained comparable CFU/ml (approximately 10^9/ml) and were in early logarithmic phase. At this phase of growth, no in vitro bactericidal activity was demonstrable in the filtered supernatant fluids of all three staphylococcal cultures. Controls of fresh TSB and a filtered supernatant fluid of a culture of strain C55 (phage Type 71) at the same phase of growth were also mixed with equal volumes of the M Type 57 culture. From the various mixtures, 0.1 ml samples were injected into the backs of hamsters. By 24 hr, the experimental lesions had developed into

[1] *Abbreviations used in this paper:* CFU, colony-forming units; TSB, tryptic soy broth.

the crusted stage (15). At this time, cultures were taken from each lesion and the number of viable streptococci determined.

The results are shown in Fig. 1. The circles in each panel represent colony counts of surviving streptococci in individual lesions produced under identical conditions and the lines are the means for the groups. All lesions in the TSB control group (first panel) contained large numbers of viable streptococci. The frequency and numbers of streptococci recovered from mixed lesions containing

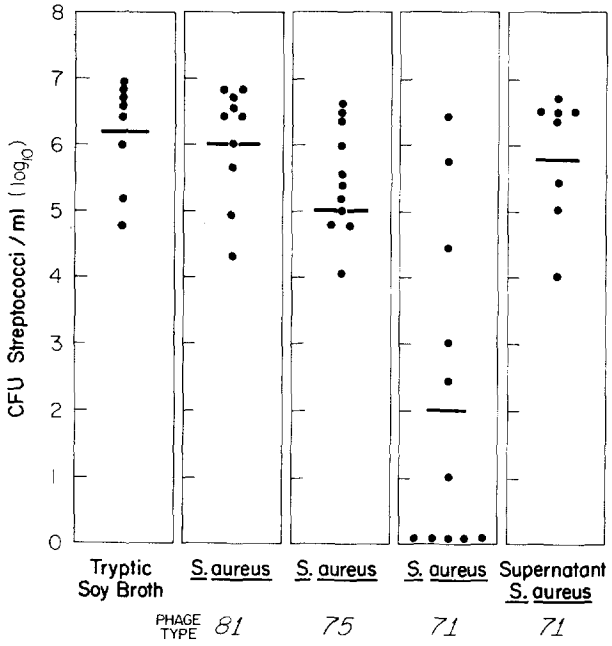

FIG. 1. Effect of whole cultures or culture supernates of three strains of staphylococci representing different phage types on recovery of Type 57 streptococci from mixed lesion.

a strain of phage Type 81 staphylococci (second panel) were comparable to the TSB control. The strain of phage Type 75 staphylococci tested (third panel) resulted in a moderate reduction in the average number of viable streptococci recovered; however, streptococci were present in all lesions. With the prototype strain of phage Type 71 staphylococci a marked bactericidal effect was often, but not invariably, demonstrated (fourth panel). In 6 of 11 lesions, streptococci were not detectable and of the remaining six, streptococci were significantly reduced in three. Since phage Type 71 staphylococci have been shown to produce a bactericidal substance which is detected in supernatant fluids (10), the in vivo effect of sterile supernatant fluids of phage Type 71 staphylococci when mixed with cultures of Group A streptococci and injected intradermally was

also examined (fifth panel). No effect on the recovery of streptococci from the lesions was demonstrated. This suggests that the reduction in viability noted with phage Type 71 staphylococci was not due to preformed bactericidal substance undetectable in the in vitro system.

A previous report from this laboratory (10) indicated variations among strains of different streptococcal M Types in their in vitro susceptibility to the bactericidal substance. Experiments were designed, therefore, to determine whether such variations in susceptibility can also be demonstrated in vivo as well as in vitro.

The in vitro susceptibility of the prototype strain of M Type 57 streptococcus was compared to that of an M Type 12 streptococcus by spotting twofold dilutions of a supernatant fluid from a 36 hr culture of strain C55 staphylococcus onto lawns of the two streptococcal strains. The M Type 57 strain was susceptible to a 1:4 dilution of the fluid whereas the M Type 12 strain was susceptible only to undiluted fluid. The in vivo susceptibilities of these two streptococcal strains were also compared. Logarithmic-phase organisms of the M Type 12 strain and the M Type 57 strain (1.2×10^8 CFU/ml and 1.5×10^8 CFU/ml, respectively) were each mixed with equal volumes of cultures of a strain of phage Type 71 or 75 *S. aureus*. 24 hr after intradermal injection of the various mixtures, the number of viable streptococci was determined in each lesion as described above. The results are illustrated in Fig. 2. Both streptococcal types are consistently recovered in high numbers from lesions containing the phage Type 75 staphylococcal strain. Results with the phage Type 71 staphylococcal strain varied strikingly in individual lesions but in general the bactericidal effect was more evident with M Type 57 than with Type 12 streptococci. This finding is consistant with the in vitro observations mentioned above.

In all of the previously described experiments, streptotocci and staphylococci were simultaneously injected into the hamster. Additional experiments were carried out to assess whether the superimposition of staphylococci on preexisting streptococcal lesions would result in demonstrable reduction of viable streptococci. Lesions were initiated in hamsters using M Type 57 streptococcus. 24 hr later, 0.1 ml of logarithmic-phase cultures of phage Types 71, 75, or 81 *S. aureus* was injected separately into the lesions. Cultures of the lesions were taken by calibrated loop 24 hr after the injections of staphylococci. The number of viable streptococci was determined as above and the results are shown in Fig. 3. As compared with phage Types 75 and 81 staphylococci, phage Type 71 *S. aureus* resulted in a moderate reduction in viable streptococcal CFU in some lesions, but the reduction in mean count was much less marked than in previous experiments when the staphylococci and streptococci were simultaneously injected.

In all the previous experiments, the streptococcal inocula consisted of 10^8

CFU/ml or greater as compared to staphylococcal inocula of 10^9 CFU/ml. The effect of smaller streptococcal inocula was next investigated. Previous studies in the hamster (15) have shown that the intradermal introduction of less than 10^6 CFU/ml of M Type 57 streptococci/injection site does not result in any noticeable lesion unless a foreign body is incorporated with the inoculum.

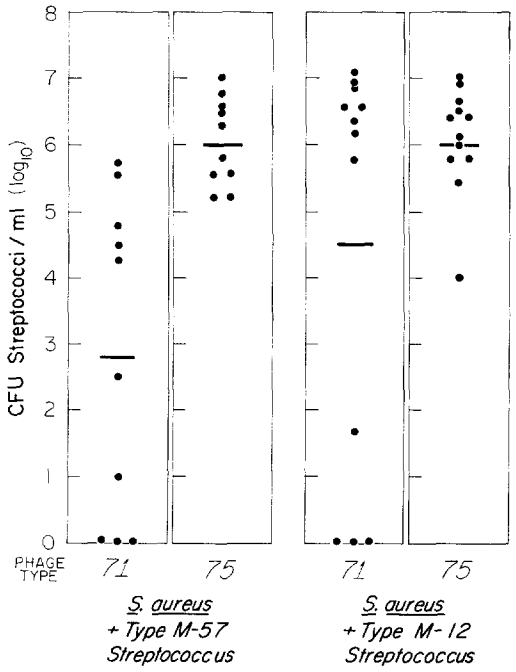

FIG. 2. Susceptibility of strains of streptococci representing two different streptococcal M Types to phage Type 71 staphylococci in mixed lesions.

Therefore, talcum powder, 1 mg/ml, was added to a logarithmic-phase culture of M Type 57 streptococci diluted to contain 10^6 CFU/ml. Equal volumes of the streptococcal suspension were added to a logarithmic-phase culture of C55 staphylococci containing 10^9 CFU/ml. A control of the same streptococcal suspension mixed with TSB was also used. Various sites were injected in hamsters and the number of viable streptococci from each lesion determined 24 hr after the injections. The results are shown in Fig. 4. In 8 of 12 lesions injected with the mixture of streptococci and staphylococci, no streptococci were recovered; however, in four lesions the numbers of streptococci recovered were comparable to the controls. As compared to the previous experiments (Figs. 1 and 2), the bactericidal effect of phage Type 71 *S. aureus* is generally more pronounced with smaller streptococcal inocula, but this effect is not universal. The bimodal dis-

tribution of results in the above experiment, which is also suggested in earlier experiments, makes the mean value nonrepresentative of the group. In view of this marked variation in the in vivo bactericidal activity in individual lesions containing identical mixtures of streptococci and phage Type 71 staphylococci, various possibilities were explored in an attempt to find a plausible explanation for this observation.

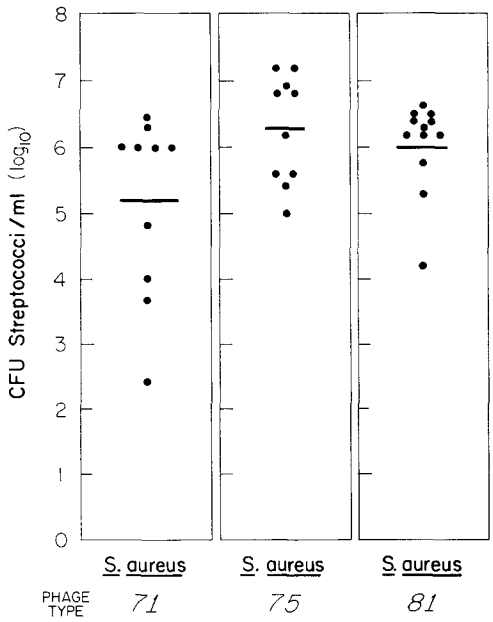

FIG. 3. Effect of delayed inoculation of phage Type 71 staphylococci into preexisting streptococcal lesions on subsequent recovery of Type 57 streptococci.

The possibility that resistant variants of streptococci might be selected in vitro or in vivo was investigated. Using lower concentrations of the bactericidal substance in the lawn-spotting method (10), colonies formed by survivors can be detected uniformly within the area of killing. Subcultures of three individual surviving colonies for each streptococcal Type (M 57 and M 12) were tested for susceptibility to the action of the bactericidal substance. All were sensitive and comparable to controls from the original frozen specimens. Also stepwise development of resistance did not occur when colonies formed by survivors were picked, subcultured, and tested for four consecutive times.

Three isolates of M Type 57 streptococci recovered from lesions where no inhibition was demonstrable were tested in vitro for susceptibility to the action of the bactericidal substance. The lawn-spotting method was employed (10). All the isolates tested were susceptible and were comparable to a nonselected

control. Thus, selection of mutants which are resistant to the action of the bactericidal substence does not seem to be the mechanism involved.

S. aureus isolates from various lesions were also studied for possible alterations in capacity to produce the bactericidal substance. An isolate from a lesion showing eradication of streptococci was compared to one from another lesion where no inhibition of streptococci occurred. Both isolates were grown separately in TSB for 36 hr, and the supernatant fluids harvested. Bactericidal activities in the filtered supernatant fluids were identical. In vivo comparison of the two staphylococcal isolates was also investigated. Equal numbers of CFU

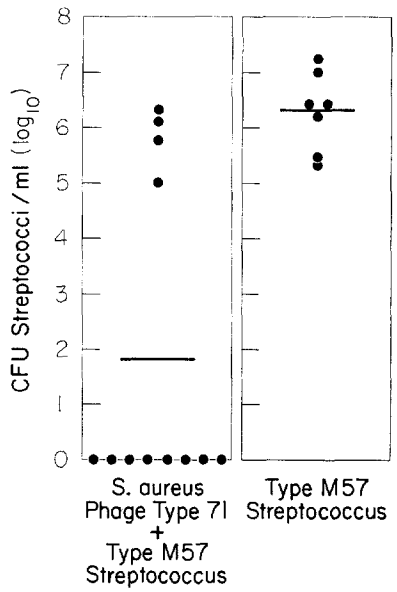

FIG. 4. Susceptibility of streptococci to the action of the bactericidal substance in lesions initiated with inocula containing talcum powder and smaller ratios of streptococci to staphylococci.

of logarithmic-phase cultures of the two staphylococcal isolates were mixed separately with M Type 57 streptococci. From each mixture, 0.1 ml samples were injected intradermally into hamsters at 16 different sites. 24 hr later the numbers of viable streptococcal CFU in the 16 lesions were determined as described previously. Results similar to those previously obtained (Fig. 1, panel 4) were observed; that is, with both staphylococcal isolates a wide scatter in the number of streptococcal CFU recovered from comparable individual lesions was again noted.

Attempts were made to demonstrate directly the presence of bactericidal activity in material recovered from lesions produced by phage Type 71 staphylo-

cocci. When tested by the lawn-spotting method, no such activity could be shown in pus or in infected skin rendered sterile by boiling for 10 min, a procedure that does not inactivate the in vitro-produced bactericidal substance. In addition, no activity could be demonstrated in similarly treated material obtained from mixed lesions in which streptococci had been eradicated by the phage Type 71 staphylococci. When bactericidal substance preformed in vitro was mixed with pieces of normal hamster skin or with excised lesions containing one or both organisms and the mixtures boiled, no reduction in the titer of bactericidal activity resulted. These findings suggest that the bactericidal substance is not inactivated by normal or infected tissues but, if produced in vivo, apparently dissipates rapidly.

DISCUSSION

Microbial antagonism is an observation of long-standing (17) and has been demonstrated both in vitro (9, 10, 18) and in vivo (19–23). Interaction among microbial agents may be a significant factor in the ecology of such flora in man. A disturbance in the balance of the normal flora is thought to contribute to certain disease processes, particularly in the gastrointestinal tract (24). Since staphylococci are common inhabitants of the respiratory tract, conjunctiva, and skin of humans, their possible role in microbial interaction has received some attention. Halbert et al. (20) have demonstrated the protective effect of *S. epidermidis* in mice against infections with *Clostridium septicum*. They have also demonstrated protection in guinea pigs against *Corynebacterium diphtheriae* infection (21). The staphylococci employed in those studies were originally isolated from human conjunctivae. *S. epidermidis* strains producing an antibiotic substance in vitro were the only staphylococci that were protective in vivo. Interference among staphylococci has been demonstrated in experimental animals (19, 23) and colonization with *S. aureus* strain 502A has been used to prevent infections or colonization with heterologous staphylococci in humans (22). Strain 502A is not a phage Type 71 or Group II staphylococcus and produces no bactericidal substance in vitro (10).

Bacterial interaction in relation to skin infections has not been investigated extensively. Anthony and Wannamaker (19) have demonstrated bacterial interference among various staphylococci in experimental burns in the rabbit. In this animal model, antagonism is not restricted to particular staphylococcal strains. The mechanism(s) by which such bacterial antagonism occurs has not been clearly elucidated in this model or in many of the other in vitro or in vivo models described. It is possible that several mechanisms exist.

The availability of an experimental model for impetigo (14, 15) has enabled the study of the interaction between staphylococci and streptococci in vivo, as reported in the present study. The results indicate that microbial antagonism between phage Type 71 staphylococci and Group A streptococci does occur in

vivo. Moreover, in this model, antagonism is not demonstrated by strains of several other staphylococcal phage types tested. The mechanism by which phage Type 71 *S. aureus* kills other organisms in vitro has been reported (12). It is tempting to assume that the same mechanism may be operable in vivo although we have no direct evidence to support this view. The relationship of the bactericidal substance from phage type 71 staphylococci to bacteriocins has been discussed at length in a previous report (11). Evidence consistent with the possibility that a well-defined staphylococcal bacteriocin may play a role in bacterial antagonism in vivo has not to our knowledge been reported previously.

Bactericidal activity could be demonstrated best when mixtures of staphylococci and streptococci were simultaneously injected into animals. Superimposition of staphylococci on preexisting streptococcal lesions resulted in a less marked bactericidal activity. The reason for this difference is not clear but could be due, at least in part, to technical difficulties in assuring adequate mixtures of organisms in vivo. In many instances, part of the superimposed staphylococcal inoculum leaked out of the lesions. The duration of contact of susceptible cells with the liberated bactericidal substance, the phase of growth of the cells, or the rate of production of the substance may also be responsible factors. Furthermore, the number of streptococcal CFU in the lesions could have increased in the 24 hr before superimposition of the staphylococci resulting in a high ratio of streptococci to staphylococci. In the animal burn model (19) a minimal time interval was required between inoculation of the interfering strain and inoculation of the challenge strain for interference to be manifested.

Variations in the in vitro susceptibility of streptococcal strains of different M Types have been noted previously (10), and similar results were obtained in vivo in the present studies. M Types 57 and 49 are more susceptible in vitro than M Types 12 and 14. The present in vivo studies demonstrate further the greater sensitivity of M Type 57 as compared to M Type 12. The biological properties of the streptococcus responsible for this difference in susceptibility require further study.

The wide variation in bactericidal effect in individual lesions produced by the same inocula is intriguing and deserves some discussion. Of particular interest is the tendency toward a bimodal distribution in the numbers of surviving streptococci, with some lesions showing no reduction in viable count and others showing complete killing of streptococci. This tendency was noted in most experiments but was particularly striking when a small streptococcal inoculum and a foreign body were used.

No explanation of this phenomenon has been obtained. Selection of resistant mutants in vitro or in vivo among M Type 57 streptococci could not be demonstrated in the present studies. No correlation could be shown between survival of streptococci in mixed lesions in the hamster and lack of production of the bactericidal substance in vitro by the staphylococci in the same lesions. Indeed,

no evidence of in vitro selection of staphylococci to become incapable of producing the bactericidal substance has been demonstrated. A possible explanation, however, is that only a few staphylococcal cells in a given population are capable of producing the bactericidal substance, some evidence for which is available in other systems. Ozeki et al. (25), using micromanipulative isolation technics, demonstrated that only 1% of a *S. typhimurium* population is capable of producing bacteriocins. Using a modification of the soft agar layer method described by these workers (25) we have been unable to demonstrate the same phenomenon with staphylococci. Irrespective of the exact explanation for this biological variation, it remains an interesting experimental finding since it is consistent with the observation that occasionally in human impetigo phage Type 71 staphylococci are recovered along with streptococci from the same lesion (1, 2, 5, 6).

SUMMARY

The interaction between staphylococci and Group A beta hemolytic streptococci in mixed lesions was investigated in an experimental impetigo model. A strain of staphylococcus of phage Type 71, which has been shown in vitro to produce a bacteriocin for streptococci and other Gram-positive organisms, eliminates or reduces Group A streptococci in mixed lesions. In contrast, staphylococcal strains of phage Types 75 and 81, which do not produce a demonstrable bacteriocin in vitro, exhibit no such effect.

Some variation was noted in the in vivo response of two different streptococcal M Types to the bactericidal effect of phage Type 71 staphylococci. Bacterial antagonism is more pronounced when staphylococci and streptococci are injected simultaneously into animals than when staphylococci are superimposed on preexisting streptococcal lesions.

Marked variations were found in the numbers of viable streptococci (colony-forming units) recovered from individual lesions containing identical mixtures of streptococci and phage Type 71 staphylococci. The frequency of a demonstrable bactericidal effect was related to the number of streptococci injected. With small inocula of streptococci, the tendency towards an all-or-none effect was particularly striking. No evidence of selection of streptococcal or staphylococcal mutants which might explain this phenomenon was obtained.

These observations suggest that the bactericidal effect of phage Type 71 staphylococci on other Gram-positive organisms, previously demonstrated in vitro, appears to operate also in vivo.

The authors thank Margaret Trahms and Judy Jaqua for technical assistance.

BIBLIOGRAPHY

1. Parker, M. T., A. J. H. Tomlinson, and R. E. O. Williams. 1955. Impetigo contagiosa. The association of certain types of *Staphylococcus aureus* and of *Streptococcus pyogenes* with superficial skin infections. *J. Hyg.* **53**:458.

2. Barrow, G. I. 1955. Clinical and bacteriological aspects of impetigo contagiosa. *J. Hyg.* **53:**495.
3. Markowitz, M., H. D. Bruton, A. G. Kuttner, and L. E. Cluff. 1965. The bacteriologic findings, streptococcal immune response, and renal complications in children with impetigo. *Pediatrics.* **35:**393.
4. Anthony, B. F., L. V. Perlman, and L. W. Wannamaker. 1967. Skin infections and acute nephritis in American Indian children. *Pediatrics.* **39:**263.
5. Dajani, A. S., F. S. Farah, and A. K. Kurban. 1968. Bacterial etiology of superficial pyoderma in Lebanon. *J. Pediat.* **73:**431.
6. Dillon, H. C. 1968. Impetigo contagiosa: suppurative and nonsuppurative complications. I. Clinical, bacteriologic and epidemiologic characteristics of impetigo. *Amer. J. Dis. Child.* **115:**530.
7. Van Toorn, M. J. 1961. On the staphylococcal and streptococcal etiology of impetigo. *Dermatologica.* **123:**391.
8. Wannamaker, L. W. 1970. Differences between streptococcal infections of the throat and of the skin. *New Engl. J. Med.* **282:** 23–31 and 78–85.
9. Parker, M. T. 1958. Some cultural characteristics of *Staphylococcus aureus* strains from superficial skin infections. *J. Hyg.* **56:**238.
10. Dajani, A. S., and L. W. Wannamaker. 1969. Demonstration of a bactericidal substance against β-hemolytic streptococci in supernatant fluids of staphylococcal cultures. *J. Bacteriol.* **97:**985.
11. Dajani, A. S., E. D. Gray, and L. W. Wannamaker. 1970. Bactericidal substance from *Staphylococcus aureus*. Biological properties. *J. Exp. Med.* **131:**1004.
12. Dajani, A. S., E. D. Gray, and L. W. Wannamaker. 1970. Effect of bactericidal substance from *Staphylococcus aureus* on Group A streptococci. I. Biochemical alterations. *Infec. Immunity.* **1:**485.
13. Clawson, C. C., and A. S. Dajani. 1970. Effect of bactericidal substance from *Staphylococcus aureus* on Group A streptococci. II. Structural alterations. *Infec. Immunity.* **1:**491.
14. Cushing, A. H., and E. A. Mortimer. 1970. A hamster model for streptococcal impetigo. *J. Infec. Dis.* **122:** 224.
15. Dajani, A. S., and L. W. Wannamaker. 1970. Experimental infection of the skin in the hamster simulating human impetigo. I. Natural history of the infection. *J. Infec. Dis.* **122:**196.
16. Dajani, A. S., P. L. Hill, and L. W. Wannamaker. Experimental infection of the skin in the hamster simulating human impetigo. II. Assessment of various therapeutic regimens. *Pediatrics.* In press.
17. Rosebury, T. 1962. Microorganisms Indigenous to Man. McGraw-Hill Book Co., New York.
18. Ribble, J. C. 1967. A mechanism of bacterial interference in vitro. *J. Immunol.* **98:**716.
19. Anthony, B. F., and L. W. Wannamaker. 1967. Bacterial interference in experimental burns. *J. Exp. Med.* **125:**319.
20. Halbert, S. P., C. Sonn, and L. Swick. 1954. Mixed bacterial infections in relation to antibiotic activities. I. *Clostridium septicum*-micrococcus infections. *J. Immunol.* **73:**169.

21. Halbert, S. P., C. S. Kazar, and L. S. Swick. 1957. Mixed bacterial infections in relation to antibiotic activities. III. Staphylococcal-diphtheria infections in guinea pigs. *A.M.A. Arch. Ophthalmol.* **57:**716.
22. Boris, M., T. F. Sellers, Jr., H. F. Eichenwald, J. C. Ribble, and H. R. Shinefield. 1964. Bacterial interference: protection of adults against nasal *Staphylococcus aureus* infection after colonization with a heterologous *S. aureus* strain. *Amer. J. Dis. Child.* **108:**252.
23. Ribble, J. C., and H. R. Shinefield. 1964. Bacterial interference in experimental staphylococcal infections. *J. Pediat.* **65:**1047.
24. Lee, A., J. Gordon, C. J. Lee, and R. Dubos. 1971. The mouse intestinal microflora with emphasis on the strict anaerobes. *J. Exp. Med.* **113:**339.
25. Ozeki, H., B. A. D. Stocker, and H. De Margerie. 1959. Production of colicine by single bacteria. *Nature* (*London*). **184:**337.