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Staphylococcidal Capability of Rabbit Peritoneal Macrophages in Relation to Infection and Elicitation: Induction and Elicitation of Activated Macrophages

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The capability of macrophages to inactivate ingested staphylococci can be augmented when repeated infection is followed by specific elicitation with staphylococcal lysate. The increase in staphylococcidal capability with specific elicitation after infection is not dramatic but is statistically significant. The percentage of change in staphylococcidal capability after infection and specific elicitation is systematically related to the staphylococcidal capability of the populations of macrophages in the same rabbits studied prior to infection. When the capability of the initial populations of macrophages has been high, the percentage of change after infection and elicitation may be slight or even negative. When the staphylococcidal capability of the initial population of macrophages in a given rabbit has been low, there is typically a significant increase in this capability after infection and elicitation. It is shown at the cellular level that it is possible to evoke a population of activated macrophages, by a procedure which is analogous to procedures reported as useful in human practice.

In seeking procedures to evoke populations of macrophages with augmented capability to inactivate staphylococci, we have been guided by two types of experience: (i) studies of cross-immunity in diseases in which induction and elicitation occur in the course of infections in experimental animals. A brilliant series of such studies in listeriosis (9), brucellosis (10), tuberculosis (10), and salmonellosis (3) has been and is being conducted by investigators at the Trudeau Institute (11).

(ii) The second is experience of a collaborating physician, Arthur G. Baker, who has used staphylococcal phage lysate clinically over a number of years and many hundreds of cases. This experience has been recorded as successful in the majority of instances (1, 2) [see also Salmon and Symonds (17)] and is presently followed in a successful clinical practice.

In applying the Trudeau experience to staphylococcal infection, it is relevant that *Staphylococcus aureus* does not multiply in macrophages (18, 19). The intracellular staphylococci are gradually inactivated, however, at rates which we find can be influenced by specific induction and elicitation. *Mycobacterium tuberculosis* (8), the *Brucellae*, and *Listeria monocytogenes* do multiply within the macrophages of unprotected animals, and the *Salmonellae* multiply within the peritoneal cavities of infected animals (3, 21). Thus specific induction and elicitation proceed concurrently in these diseases and cause activation of the nonspecific effector mechanism, the "angry macrophages". In contrast to the above diseases, procedures against staphylococci must include specific elicitation by staphylococcal antigens, applied by the investigator in animals or in the patient by the physician.

MATERIALS AND METHODS

Organism. *S. aureus* strain 18Z was used throughout as test organism and for infection of rabbits and was prepared and used as described in the previous report (7).

Phage-lysed staphylococci. Staphylococcal phage lysate (SPL; Staphage lysate, Delmont Laboratories, Inc., Swarthmore, Pa.) was used for skin testing and for treatment of rabbits after infection with *S. aureus* (7).

Skin tests. Skin tests were performed in the ear with 0.1 ml of SPL administered intradermally during the week following the last infection with *S. aureus*, as described in the preceding paper.

Elicitation with SPL. Also during the week after the last infection with *S. aureus*, two to three subcutaneous injections of 0.1 to 0.2 ml of SPL were administered. Peritoneal macrophages were then obtained and tested the following week. Some rabbits

receiving the above treatment received a further course of 0.1 ml of SPL once a week for 5 to 6 weeks. These were again skin-tested and given the two to three subcutaneous injections of 0.1 to 0.2 ml of SPL, and their macrophages were also tested the week after the last injection of SPL.

The schedule most commonly followed was: Monday, 0.1 ml of SPL intracutaneously as skin test; Wednesday, Thursday, and Friday, 0.2 ml of SPL subcutaneously as eliciting injections. Tuesday or Wednesday of week after, peritoneal macrophages were withdrawn. The above schedule was adopted to obtain the activated macrophages, thus avoiding the macrophage disappearance phenomenon described by D. S. Nelson (15).

Testing of staphylococcidal capability. The modified Maaløe and tissue culture-chamber methods were used as described (7).

RESULTS

The variability in staphylococcidal capability of the peritoneal macrophages of different rabbits posed a methodological problem, as already mentioned (7). The ability to inactivate staphylococci has, therefore, been measured in populations of macrophages from individual rabbits before and after infection and elicitation. Means of survival curves of staphylococci in groups of rabbits initially and after infection and elicitation have been compared, and also scatter plots of results from individual rabbits have been constructed and analyzed.

Macrophages from a series of 20 rabbits were tested by the modified Maaløe method. Rabbits were then infected with *S. aureus* 18Z for 5 weeks, skin-tested, and treated with SPL during the sixth week. The resulting populations of macrophages were tested during the seventh week. At the same times, macrophages from a series of 15 untreated rabbits were also tested as normal controls to determine the effects of factors, other than infection and elicitation, such as intraperitoneal injections of mineral oil and aging of the rabbits.

Figure 1 shows the mean results of viable total, cell-bound, and supernatant fractions of *S. aureus* per ml at the sampling times of 0, 30, 60, 120, 180 min. Figure 1A shows results of rabbits prior to and following infection with *S. aureus* and treatment with SPL. Figure 1B shows results of normal control rabbits. In the treated group, the increases in killing of the total *S. aureus* at the 120- and 180-min sampling times, while small, are statistically significant at the 0.05 and 0.025 levels, respectively. No statistically significant difference occurred in the control group at any of the sampling times.

Results from both tests on each individual rabbit were then considered. Figure 2A shows a scatter plot of results at 180 min for the 20 treated animals. The per cent survival of *S. aureus* in macrophages from each animal before treatment is represented on the abscissa and the per cent difference (increase or decrease) after treatment is represented by the ordinate. The plot of the estimating equation $Y_c = a + b \log X$ is also shown. As can be seen in this figure, a definite correlation exists between the killing before treatment and the difference in killing after treatment. The coefficient of correlation after treatment using results from all 20 rabbits is 0.841. When the

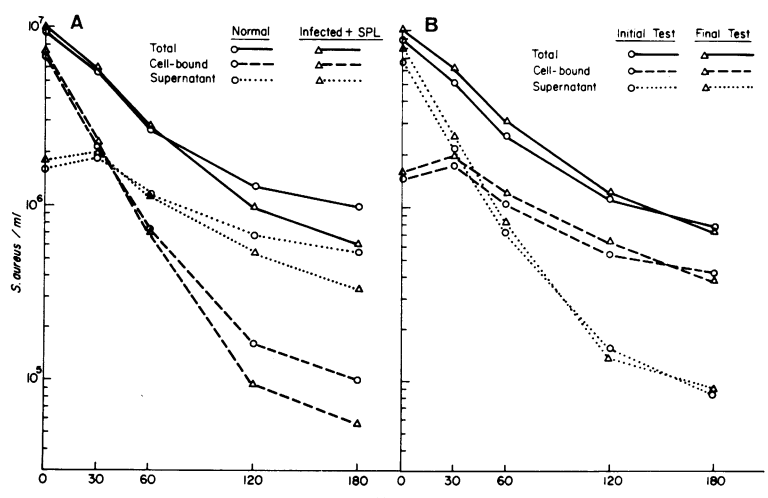

FIG. 1. *The means of total, cell-bound, and supernatant surviving Staphylococcus aureus per milliliter using the modified Maaløe method. A. Macrophages from a group of 20 rabbits before and after infection with S. aureus and elicitation with SPL. B. Macrophages from a group of 15 rabbits at the same time intervals as the above but without treatment by infection or SPL.*

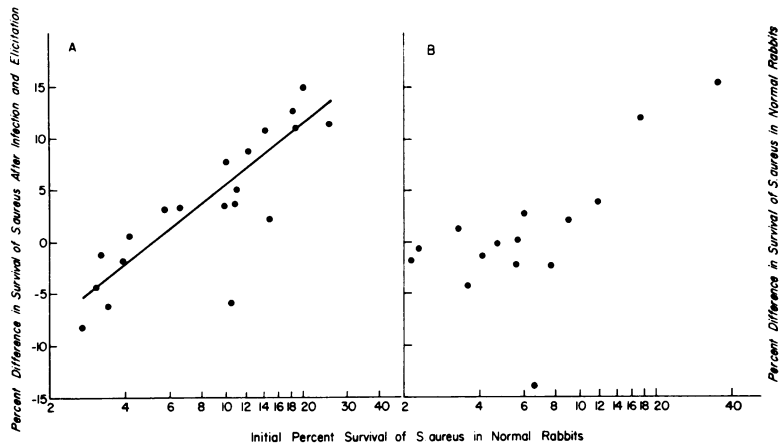

FIG. 2. *Scatter plots of results at 3 hr from individual rabbits using the Maaløe method. The abscissae are the per cent survivals of Staphylococcus aureus in the same rabbits. The ordinates are the per cent increase or decrease in survival of S. aureus in the same rabbits, (A) following infection with S. aureus and elicitation with SPL, and (B) after the same length of time as in A but without infection or elicitation. Coefficients of correlation for A = 0.92, for B = 0.69. The estimating equation $Y_c = a + b \log X$ is plotted as a line. Coefficients of correlation and probabilities of significance are calculated from the following formulas:*

Coefficient of correlation $r =$

$$N \sum (\log X \cdot Y) - (\sum \log X)(\sum Y) / \sqrt{[N \sum (\log X)^2 - (\sum \log X)^2][N \sum Y^2 - (\sum Y)^2]}$$

Probability: $t_{N-2} = \sqrt{(N-2)(r^2)/1 - r^2}$

values represented by the point furthest from the line (lower, center) are omitted, the coefficient is 0.916. Both of these coefficients were found to be statistically significant ($P < 0.001$). Thus the largest increase in killing ability is related to a low initial ability, and the largest decrease is related to a high initial ability. In the majority of these rabbits, killing ability increased after infection with *S. aureus* and elicitation with SPL.

This phenomenon is not generally shown by the normal control rabbits in which differences in three-quarters of the animals ranged only from plus to minus 3% (Fig. 2B). This figure shows the same type of scatter plot for the normal control animals with the per cent difference representing the difference between the first and second tests on the individual untreated animals. The single dot in the upper right corner represents one rabbit whose monocytes had an extremely low initial killing but showed a decided increase in per cent killing on the second test. Omitting this point and the very low point, the correlation coefficient for the other observations is 0.69 which was found to be significant at the 0.01 level. When the values for the other high point are omitted the coefficient of correlation drops to 0.465 ($P = 0.1$). Thus, when the initial killing ability has been very low, increases may occur due to causes other than experimental infection and elicitation.

A small group of six rabbits, given the above treatment of *S. aureus* infection and SPL, were given a longer course of 0.1 ml of SPL once a week for 5 to 6 weeks. During the following week, they were again skin-tested and received 0.1 ml of SPL two to three times. Their macrophages were then tested by the Maaløe method 4 to 6 days after the last injection of SPL. Three of the rabbits in the first series were also included in this series. As can be seen in Fig. 3, the increase in killing can be maintained by the above course of treatment.

Macrophages from 15 of the 20 infected and elicited rabbits, and 10 of the 15 untreated rabbits tested by the modified Maaløe method, were also used in the tissue culture-chamber method (Fig. 4). Since, in many cases, staphylococci were too few in number to give accurate bacterial counts at the 44- and 48-hr samples, results of mean *S. aureus* per macrophage are given only through the 27-hr sample. Increases in killing ability did occur following infection and elicitation. Whereas these differences in staphylococcidal capability are not statistically significant at the 0.05 level, significant differences can be noted when these results are compared with those after infection with *S. aureus* without elicitation at the different sampling times (Table 1).

When the results from each rabbit were con-

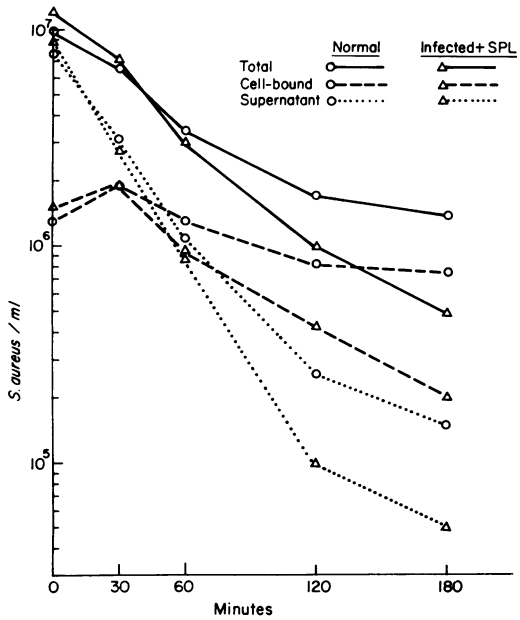

FIG. 3. *The means of total, cell-bound, and supernatant Staphylococcus aureus per milliliter using the Maaløe method. Macrophages from a group of six rabbits before and after infection with S. aureus and a continuing 6- to 7-week course of injection of SPL.*

sidered individually, 11 of the 15 treated rabbits and only 3 of the control rabbits showed increases at the 20- to 48-hr sampling times. Figure 5 shows scatter plots of the individual differences compared with the initial killing ability as tested by the tissue culture-chamber method. Figure 5A shows results from rabbits infected with *S. aureus* and elicited with SPL, and 5B shows results from the normal control rabbits. Results at 27 hr were used because in some cases too few organisms remained at the 44- and 48-hr sampling times to give reliable bacterial counts.

Again, as with the Maaløe method, a correlation can be noted between the per cent survival and the per cent difference in survival after infection and elicitation. The coefficient of correlation for this group is 0.891, which is statistically significant ($P < 0.001$). Here, again, the largest increases in killing ability are related to low initial ability and decreases to a high initial ability.

With the nine normal control rabbits (omitting the point in the lower right corner; Fig. 5B), the coefficient of correlation is 0.768 ($P = 0.005$). However when the uppermost point is omitted, the coefficient drops to 0.562 ($P > 0.1$). Thus, with this method, as with the Maaløe method, when initial killing has been very low, increases may occur due to causes other than experimental infection and elicitation.

As a comparison between the two methods used for testing, Fig. 6 shows scatter plots with the per cent difference in killing ability at 3 hr as tested by the Maaløe method represented on the abscissa and the per cent difference in killing in the chamber method at 27 hr on the ordinate for each individual rabbit. The coefficient of correlation for the 15 rabbits tested by both methods

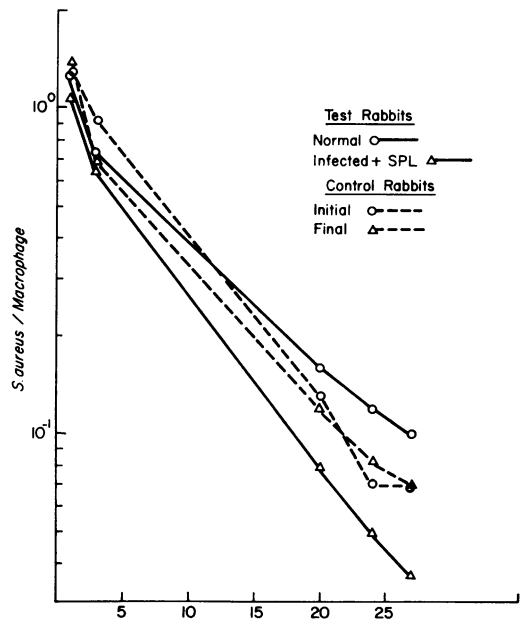

FIG. 4. *The means of surviving S. aureus/macrophage using the tissue culture-chamber method. Macrophages were from a group of 15 rabbits before and after infection with S. aureus and elicitation with SPL and from a second group of 10 rabbits at the same time intervals as the above but without treatment by infection and SPL.*

TABLE 1. *Mean per cent differences in staphylococcidal capability as determined by the tissue culture-chamber method*[a]

Sampling times (hr)	Infected[b]	Infected[b] + SPL	Probability
3–4	−4.36	−1.18	>0.7
20	−8.58	+2.08	0.025
24	−6.44	+2.41	0.025
27	−6.20	+2.65	0.02
44	−1.40	+1.38	0.05
48	−1.08	+1.06	0.10

[a] A comparison of mean per cent differences after infection with those after infection and elicitation. The *t* test was used to determine the significance of differences between the two groups.
[b] With *Staphylococcus aureus*.

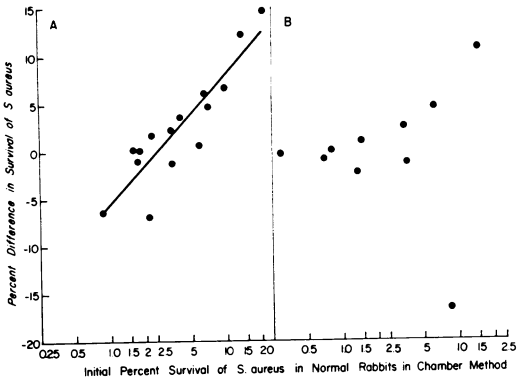

FIG. 5. *Scatter plots of results at 27 hr from individual rabbits with the tissue culture-chamber method. The abscissae are the per cent survivals of S. aureus in macrophages from individual normal rabbits. The ordinates are the per cent increase or decrease in survival of S. aureus in the same rabbits; A, following infection with S. aureus and elicitation with SPL; and B, after the same length of time as in A but without infection and elicitation. Coefficients of correlation A, 0.89; B, 0.77. r =*

$$\frac{N \sum (\log X \cdot Y) - (\sum \log X)(\sum Y)}{\sqrt{[N \sum (\log X)^2 - (\sum \log X)^2][N \sum Y^2 - (\sum Y)^2]}}$$

Probability: $t_{N-2} = \sqrt{(N-2)(r^2)/(1-r^2)}$

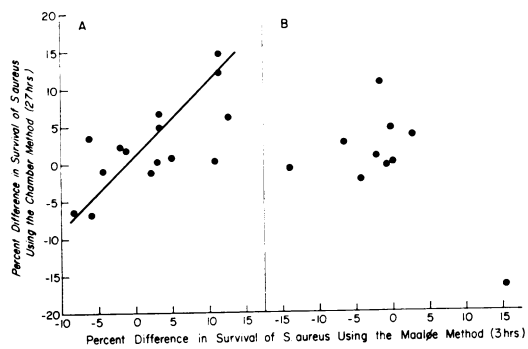

FIG. 6. *Scatter plots comparing the % difference in survival of S. aureus at 3 hr using the Maalφe method with the % difference at 27 hr using the tissue culture-chamber method. A, Following infection with S. aureus and elicitation with SPL; B, after the same length of time as in A but without treatment. Coefficients of correlation A, 0.70, B, 0.15. Coefficients of correlation and probability of significance are calculated from the following formulas: Coefficient of correlation r =*

$$\frac{N(\sum XY) - (\sum X)(\sum Y)}{\sqrt{[N \sum X^2 - (\sum X)^2][N \sum Y^2 - (\sum Y)^2]}}$$

Probability: $t_{N-2} = \sqrt{(N-2)(r^2)/(1-r^2)}$

before and after infection and elicitation (Fig. 6A) is 0.704, which is statistically significant ($P < 0.005$). The coefficient for the nine normal control rabbits (omitting the point in the extreme lower right) of Fig. 6B is 0.149 ($P > 0.70$).

The results of the two methods used for testing the intracellular killing of *S. aureus* are in essential agreement. It appears that elicitation with SPL after a series of infections with *S. aureus* produced significant increases in the killing ability of macrophages when results were averaged. When results from individual rabbits were compared, differences were related to the initial ability of the macrophages from the same animal before treatment. In contrast, the differences shown in the normal control rabbits were so small as to be negligible when results were averaged and were not generally correlated to the initial ability of the macrophages when results from the two tests on each individual rabbit were compared.

DISCUSSION

S. aureus, in its immune reactions, differs in important respects from other pyogenic pathogens (12, 13, 20). Infection with *S. aureus* evokes allergy, characterized by delayed-type hypersensitivity (7), and fortunately this allergy is found to provide a basis upon which specific elicitation with staphylococcal antigens can augment staphylococcidal capability.

The survival curves of staphylococci before and after infection and elicitation afford evidence of augmented staphylococcidal capability of the peritoneal macrophages, which is significant, if not dramatic.

Analysis of the scatter plots provides other evidence. The macrophage populations found after infection and elicitation must have been evoked by a whole series of interactions and transformations (7).

The change in killing capability of the peritoneal macrophages was measured by comparison of the capability after infection and elicitation with the initial capability. Since the denominator in this ratio, the initial capability, can vary from high to low, some such relationship between final and initial killing capability as we have found was to have been expected. The estimating equations for the correlation between initial and final killing capabilities were plotted, and the coefficients of correlation with the experimental values were found to be high. For untreated rabbits, the correlation coefficients were much lower. Infection and elicitation must, therefore, have evoked populations of augmented staphylococcidal capability.

Allergy to *S. aureus* expressed as delayed-type skin hypersensitivity exists in a high proportion of

human beings, doubtless due to colonization, subclinical or clinical infection, with *S. aureus* (14). This allergy is a mixed blessing. In and of itself it does not increase resistance. It does, however, afford a basis upon which elicitation with staphylococcal antigens can eventuate in activation of macrophages and increased resistance. The effector mechanism, i.e., the activated macrophage, is known to be nonspecific in efficacy.

In these communications, we have measured activation of macrophages in terms of their staphylococcidal capability, thus, in terms of resistance at the cellular level against the pathogen which originally infected the animals. The fact that specific induction and elicitation evoke activation of the macrophages as a nonspecific effector mechanism is firmly established by the brilliant investigations at the Trudeau Institute (3, 9–11) and elsewhere (6, 20; J. B. Hibbs et al., Eighth Annu. Meet. Reticuloendothel. Soc., 1972, *in press*). However in the Trudeau work, induction and elicitation occurred through the medium of experimental infections by *Mycobacterium tuberculosis*, the *Brucellae*, *Salmonellae* or *Listeria monocytogenes*. The contribution we hope to make is in showing that induction such as occurs naturally in human subjects (14) followed by elicitation can evoke increased resistance (7). Such induction and elicitation by the H37Ra-O.T. system and the *S. aureus*-SPL system has also been shown to afford significant protection in vivo against challenge with vaccinia virus (E. G. Allen and S. Mudd, *manuscript in preparation*).

The principle of specific induction and elicitation resulting in nonspecific resistance is well established. We believe we have shown that this principle can be used to evoke resistance at the cellular level, with means which can be and are being used in clinical practice (1, 2, 14, 17). What we have not determined, and which remains as a major challenge, is the range of efficacy of specific induction and elicitation in affording nonspecific resistance in significant human situations. Such significant situations include, in principle, the common cold and influenza and such major plagues as leprosy and trachoma. Will reticuloendothelial activation prove adequate to afford significant relief in such conditions?

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