

# Staphylococcal Capability of Rabbit Peritoneal Macrophages in Relation to Infection and Elicitation: Delayed-Type Hypersensitivity Without Increased Resistance

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The staphylococcal capability of populations of peritoneal macrophages in rabbits has been measured before and after repeated infections with *Staphylococcus aureus*. Such rabbits after infection showed delayed-type hypersensitivity to *S. aureus* antigens, but the staphylococcal capability of the peritoneal macrophages was not increased. This result at the cellular level is in agreement with previous assessment in vivo of the consequences of staphylococcal infection. Pathways to cell-mediated resistance, with and without delayed-type hypersensitivity, are presented.

The reality and the importance of cell-mediated resistance are well established. In relation to the rejection of tissue and organ transplants, cell-mediated resistance has required suppression. In relation, however, to a number of infectious diseases, of which tuberculosis is the best studied, cell-mediated resistance is a means of prevention and the major component in natural recovery. Brucellosis and salmonellosis are now under intensive investigation as infections in which cell-mediated and humoral immunity play complementary roles.

To the team of which one of us is responsible investigator, it has for some time seemed opportune that major efforts be directed toward putting cell-mediated resistance to work by means which are practicable in human medicine. In fact, we believe such application has already been made without, however, complete understanding of the mechanisms involved (2, 3, 20, 23, 28).

Induction of allergy by infection followed by elicitation with homologous antigen is known to activate the reticuloendothelial system, and the effector mechanism, the activated or "angry" macrophages, is known to be nonspecific in its capability to inactivate intracellular pathogens. A high proportion of human subjects have a naturally induced allergy to *Staphylococcus aureus*, doubtless due to colonization, subclinical and clinical infections, with *S. aureus* (20). Elicitation by staphylococcal antigens can be and is being practiced in such human subjects (2, 3,

28). These facts suggest that specifically induced allergy to *S. aureus* and specific elicitation with staphylococcal antigens may afford a favorable opportunity to put cell-mediated resistance to work in defense against a number of appropriate infections, including some due to viruses. Parenthetically it may be added that the history of vaccination from Edward Jenner (10) on is one classic example of the importance of cell-mediated immunity in a virus disease.

In earlier work it has been shown that local resistance to challenge with *S. aureus* can be evoked by induction by staphylococcal infection and elicitation with a mixture of staphylococcal antigens (30). A certain ambiguity, however, was inherent in this work, in that challenge was at the site in which a granuloma had been evoked by the previous elicitation. Was the local resistance due to the number of macrophages mobilized at this site, to the augmented staphylococcal capabilities of the individual macrophages, or to both?

To clarify the role of the committed reticuloendothelial cells themselves, it seemed necessary to determine the staphylococcal capability of peritoneal exudative cells in vitro. Rabbits were chosen as the experimental animals. Early observations (27) showed that macrophages from different rabbits differed significantly in their staphylococcal capabilities. Thus it became important to compare the staphylococcal capabilities of peritoneal exudative cells from

each animal before the experiment and after infection or infection and elicitation had been practiced. A systematic relationship of initial staphylococcal capability to that following infection alone, and to infection and elicitation was thus disclosed.

### MATERIALS AND METHODS

**Organisms.** *S. aureus* strain 18Z, first described by Kapral and Shayegani (12) was used throughout as a test organism and to produce staphylococcal infections in rabbits. Strain 18Z is of relatively low virulence (5). Eighteen-hour cultures were grown in Trypticase soy broth (TSB; Baltimore Biological Laboratories) and were washed twice with 0.85% saline containing 1% TSB before use.

**Experimental procedures.** A suspension (0.1 ml) of *S. aureus* 18Z containing  $10^9$  colony-forming units (CFU) was injected intradermally into each side of market rabbits at 1-week intervals for a period of 5 weeks. This procedure was subsequently found to be sufficient to produce a delayed-type hypersensitivity by the beginning of the sixth week, as shown by skin tests in the ear (Fig. 3).

**Skin tests.** In one series of rabbits, skin tests were done in the ear, with 0.1 ml of staphylococcal phage lysate (SPL, Staphage lysate, Delmont Laboratories, Inc., Swarthmore, Pa.) in the week following the last infection with *S. aureus*. This is a product containing the lysate of  $10^8$  to  $5 \times 10^8$  CFU of *S. aureus* of serological types I and III, and  $10^8$  to  $10^9$  plaque-forming units of polyvalent staphylococcal bacteriophage per ml (2, 3, 29). The amount of reaction was determined by measuring the increase in thickness of the ear with dial-gauge calipers and by measuring the area of erythema or induration, or both. Measurements were made before injection and immediately after injection and at 30 min, 2 hr, and 24 hr. Since the smallest value of ear thickness after injection occurred at the 2-hr reading, final results were obtained by subtracting the 2-hr reading from the 24-hr reading and multiplying this result by the length times the width of erythema or induration. No immediate reactions occurred in any of the infected rabbits. Beef heart infusion, the culture medium used in the preparation of SPL, was used as a control in the other ear. Normal rabbits were also skin-tested in the same manner.

**Exudative cells.** Rabbit peritoneal exudates were obtained by injecting 30 ml of mineral oil into the peritoneal cavities. Four to six days later, 200 to 300 ml of Hanks balanced salt solution (BSS) containing 1% heparin was injected into the peritoneal cavity and mixed by kneading the abdomen. The mixture of cells, predominantly macrophages with some lymphocytes and granulocytes, oil, and Hanks solution were then withdrawn with a syringe and 18-gauge needle. Cells were washed twice with Hanks BSS, and the number of macrophages was determined by counting in a hemacytometer. Animals remained alive so that the same animal could be tested for staphylococcal capability after infection for comparison with the test before infection.

**Serum.** In all experiments, serum and macrophages were obtained on the day of testing from each rabbit. Blood for serum was obtained by cardiac puncture.

**Intracellular fate of *S. aureus*.** Tissue culture-chamber method: In this method described by Kapral and Shayegani (12), macrophages were mixed with 10% fresh autologous serum and 25 *S. aureus* CFU per macrophage. This suspension was then diluted with Hanks BSS to give a concentration of  $10^7$  macrophages/6 ml, the amount to be added to each tissue culture-chamber containing cover slips.

At the end of this period, the fluid was removed and the chambers were washed two to three times with Hanks BSS. Each chamber then received 6 ml of medium containing 10% fresh autologous serum, 300  $\mu$ g of streptomycin, and 0.1 ml of 10% sodium bicarbonate in Hanks BSS. Chambers were then incubated at 37 C in 5% CO<sub>2</sub> in air. Samples were removed at intervals of 2, 4, 19 to 20, 24, 27, 44, and 48 hr after the addition of medium. At each sampling time, two cover slips and 0.5 ml of medium were removed. One cover slip was used for a macrophage count, the other was ground with 1 g of Ballotini and 1 ml of 5% saponin solution. This ground mixture was plated out in serial dilution to determine the number of intracellular CFU units of *S. aureus*. The saline used for all serial dilutions contained 0.85% NaCl and 1% Trypticase soy broth. The ratio of intracellular *S. aureus* per macrophage was determined for each sampling time. The 0.5-ml sample of medium was also serially diluted and plated out to check the efficacy of the streptomycin to limit the amount of extracellular growth. In almost all cases, tests were done in duplicate or triplicate chambers.

**Modified Maaløe method.** In the modified Maaløe method (24), the macrophages and *S. aureus* were prepared as above. A total of  $3 \times 10^7$  macrophages, 10% fresh autologous serum, and  $3 \times 10^7$  *S. aureus* organisms were mixed in a total volume of 3 ml with Hanks BSS, in most cases in triplicate. These were incubated at 37 C on a roller.

Samples were taken immediately upon addition and mixing of *S. aureus* and at 30, 60, 120, and 180 min thereafter. At each sampling time, 0.2 ml was added to 0.8 ml of 5% saponin, which was then serially diluted with saline and plated to determine the total numbers of viable *S. aureus*. At the same time, 0.2 ml was added to 1.8 ml of cold saline, centrifuged for 4 min at 500 rev/min, and the supernatant fluid was serially diluted and plated to determine the extracellular count. The sediment was mixed with 1 ml of saponin, diluted, and plated to determine the cell-bound count of *S. aureus*. One-tenth milliliter of sample also was added to trypan blue to check the viability of the leukocytes.

### RESULTS

**Variability in capability of macrophages to inactivate staphylococci in normal rabbits.** Populations of macrophages inactivate the cells of staphylococci they have ingested but at different rates depending upon a variety of circumstances (25, 26). Figure 1 shows the range of variation

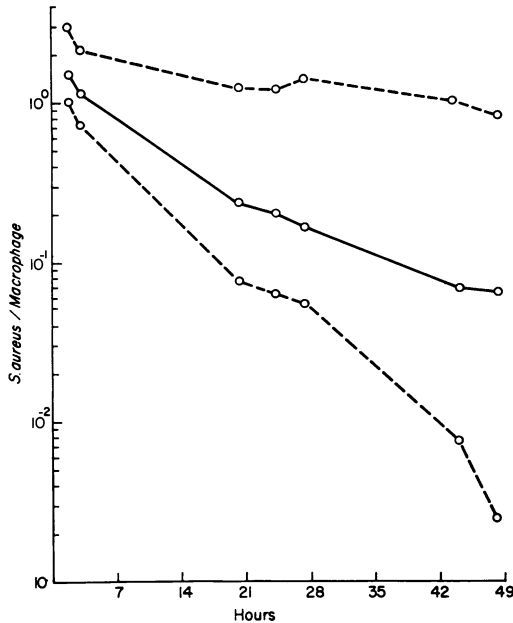


FIG. 1. Survival of *Staphylococcus aureus* per macrophage, using the tissue culture-chamber method and macrophages from 30 normal market rabbits. (-----) Highest and lowest staphylococcidal capability of individual rabbits. (—) Mean for the 30 rabbits. Standard deviations of mean at successive points, beginning at upper left and proceeding to lower right of solid line are 1.97, 1.81, 0.43, 0.40, 0.35, 0.20, and 0.17.

found in the market rabbits we have used, together with the mean survival of ingested staphylococci over a 48-hr period in 30 untreated rabbits.

This variability of different populations of peritoneal macrophages in their capability to inactivate staphylococci obviously poses a methodological problem. Our object is to determine the effect upon staphylococcidal capability, first, of repeated infections with *S. aureus* and, subsequently, of infection followed by appropriate specific elicitation (14, 19).

Max B. Lurie in his monumental investigation of the factors governing resistance to tuberculosis (15) undertook successfully the development of inbred families of rabbits of high, low, and intermediate resistance to tuberculosis (1).

Since suitable inbred strains of rabbits were not available to us, however, we had recourse to comparison of the capability of macrophages of each individual rabbit before and after repeated infections with *S. aureus* or before and after infection and specific elicitation (14). Presumably this recourse obviated genetic differences but not necessarily other differences (8).

In assessing our results, it has therefore been necessary to compare groups of rabbits in normal and infected categories. When this is done, relationships are disclosed which are interpretable.

**Staphylococcidal capability of macrophages following repeated infection.** The populations of peritoneal macrophages of 14 normal rabbits were tested by the tissue culture-chamber method as described above. Each of these rabbits was given five weekly injections of *S. aureus*, and 5 to 7 days after the last injection their macrophage populations were tested again by the same method. Autologous serum was obtained at the same time as the collection of the peritoneal macrophages.

Figure 2 shows the mean results for these 14 rabbits before and after infection. The number of staphylococci per macrophage at the 2-hr sampling time was taken as 100%. Survival of staphylococci at the other sampling times is given as percentages of staphylococci per macrophage at these times. The difference in survival between the means of normal and infected animals is not striking. However, that difference is toward a decrease in the killing capability of macrophages after infection rather than an increase.

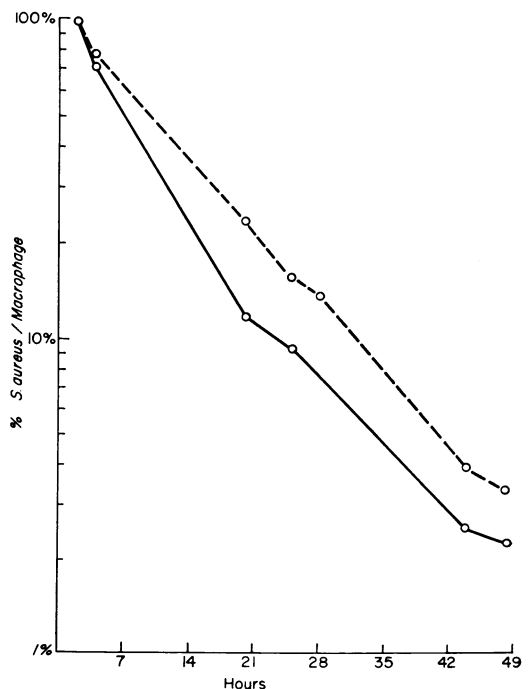


FIG. 2. Intracellular inactivation of *Staphylococcus aureus* by using the tissue culture-chamber method and macrophages from 14 rabbits before infection (—) and after five weekly infections (-----).

TABLE 1. Per cent survival of *Staphylococcus aureus* per macrophage from individual rabbits before and after infection by tissue culture-chamber method<sup>a</sup>

Rabbit no.	% Survival at 48 hr	
	Normal rabbits	% Difference after infection
1	0.12	-3.75
2	0.25	-2.81
3	0.32	-0.43
4	0.38	0.08
5	0.46	-4.73
6	0.74	-6.07
7	1.38	-0.31
8	1.41	-5.57
9	1.48	-1.04
10	1.53	0.37
11	3.59	1.91
12	4.29	2.08
13	6.54	-0.67
14	9.85	5.76

<sup>a</sup> The figures in the two columns were calculated from the following formulas: (column 2) per cent survival = (*S. aureus* per macrophage at 48 hr) per (*S. aureus* per macrophage at 2 hr). The 2-hr value was taken as 100%; (column 3) per cent difference = per cent survival per macrophage initially, minus per cent survival per macrophage after infection. Per cent differences with minus signs indicate that staphylococidal capability was decreased after infection.

Results from the individual rabbits are given in Table 1. The second column shows the per cent survival of *S. aureus* per macrophage at 48 hr in populations from rabbits before infection. The third column shows the per cent differences in survival of *S. aureus* per macrophage in populations from the same rabbits after infection. The majority of the rabbits show a decrease in staphylococidal capability. However, in general those rabbits whose macrophage populations were killing best before infection gave populations of lesser effectiveness when the populations were obtained after infection. Rabbits whose macrophage populations were not killing well initially gave populations somewhat increased in their staphylococidal capability after infection.

The probabilities of significance of difference, using the *t* test, were employed to compare the mean staphylococidal capabilities of the macrophage populations of 14 normal animals with those after infection. Probabilities of significance of differences at various times were, respectively: >0.5 at 2 hr, >0.06 at 4 hr, 0.05 to 0.10 at 20 hr, 0.05 to 0.10 at 24 hr, 0.05 to 0.10 at 28 hr, >0.1 at 44 hr, and 0.2 at 48 hr. No significant difference

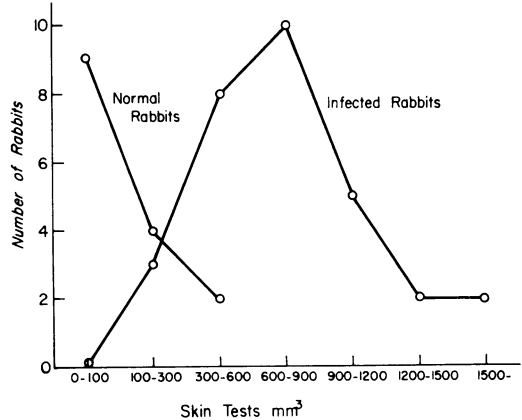


FIG. 3. Delayed-type hypersensitivity in rabbits. Frequency of distribution of the number of normal and infected rabbits showing induration of different volumes.

at the 0.05 level was found when results of all 14 animals at the various sampling times were compared with those of the same animals after infection.

The macrophage populations of normal rabbits had previously been compared by the modified Maaløe method for staphylococidal capability in the presence of normal and immunized rabbit serum. Staphylococidal capability was the same in normal and immune rabbit sera (24; Fig. 2).

**Delayed-type hypersensitivity after repeated infections.** Figure 3 shows the results of tests of delayed-type hypersensitivity in 15 rabbits before infection and in 30 rabbits after the standard course of infections with *S. aureus*. The volumes of induration in the rabbits' ears were estimated as described above. The frequency distribution within the two groups for varying amounts of delayed-type hypersensitivity is shown. The mean skin-test value was 775 mm<sup>3</sup> for the infected rabbits and 100 mm<sup>3</sup> for the untreated rabbits. It is emphasized that these rabbits had been repeatedly infected but not yet subjected to elicitation (see also 4).

## DISCUSSION

**Delayed-type hypersensitivity and cell-mediated resistance.** The relationship of skin hypersensitivity and resistance has been complicated by the fact that there is more than one type of cell-mediated resistance. A long and meticulous investigation by the Youmans (22, 37, 38) has culminated in isolation of ribonucleic acid (RNA) from the ribosomal fraction of *Mycobacterium tuberculosis* which, injected into mice, produces as effective an immunity as an equivalent amount of RNA embodied in the living cells of H37Ra tubercle bacilli. This resistance is not accom-

panied by delayed-type hypersensitivity. So far as present evidence is available, this resistance is not accompanied by resistance against *Listeria monocytogenes* or *Klebsiella pneumoniae* (38) or against *S. aureus* or *Pseudomonas aeruginosa* (De Courcy and Mudd, unpublished data). It would be of great interest to learn whether or not this resistance is exhibited against other *Mycobacteria*.

Venneman, Bigley, and Berry (33, 34) have also purified RNA from *Salmonella typhimurium*. This resistance is also not accompanied by delayed-type hypersensitivity. Its specificity is not yet defined (see reference 36).

Thompson and Snyder (31) have prepared ribosomal RNA from pneumococci type 3 which had lost its capsule. This RNA protected mice against capsulated pneumococci, types 1, 2, 3, and 7.

A ribosomal vaccine from *P. aeruginosa* at least protects the homologous strain of *Pseudomonas* (R. L. Smith et al., Eighth Annu. Meet. Reticuloendothel. Soc., 1972, in press). Specificity is under present investigation.

The pathway by which these ribosomal RNA species produce their highly potent cell-mediated immunity has not yet been defined. In the present writer's judgment, however, it is rational to suppose that a similar pathway is followed with these ribosomal immunogens and with the immunogens involved in induction and elicitation, i.e., ingestion by macrophages, liberation of "super-antigens", recognition by competent thymus-modulated lymphocytes (35), blast transformation, clonal proliferation, liberation of mediators, proliferation of monocytes, and activation of macrophages (9, 32).

In the experiments of the present study, repeated infection with *S. aureus* does result in delayed-type hypersensitivity, but until elicitation is practiced with staphylococcal phage lysate (14) increased staphylococidal capability is not found.

Mackaness and his colleagues at the Trudeau Institute, who in a brilliant series of studies have stressed the concurrence of delayed-type hypersensitivity and cell-mediated resistance, has recently written (18): "They are obviously not the same thing, even though they may be mediated by the same population of committed lymphocytes . . . activated macrophages are the direct outcome of a delayed-type hypersensitivity reaction, but the precise mechanism of macrophage activation remains obscure." Our observations are in agreement with this statement in so far as cell-mediated resistance evoked by infection and elicitation is concerned.

**The failure of infection alone to increase resistance to staphylococcal invasion.** *S. aureus* does not multiply in macrophages (25, 26), in contrast

to *M. tuberculosis*, the *Brucellae*, and *L. monocytogenes*, which do. These last pathogens produce ongoing infections in which induction and elicitation both occur in the continuing disease. Mackaness and his colleagues at the Trudeau Institute (16, 17) have shown that a maximum of resistance to superinfection tends to occur at the time when the concentration of the infecting pathogens in the tissues is maximal. Of course *S. aureus* can also produce generalized invasion, but the usual picture is of a more localized infection. It has been shown also that circulating immune antibodies are inefficacious in promoting phagocytosis and killing of *S. aureus* (24). It is perhaps understandable, therefore, that infection by *S. aureus* of itself should not set up resistance to superinfection.

Panton and Valentine (21) demonstrated that repeated infection of rabbits with *S. aureus* resulted in skin hypersensitivity. The effects upon resistance to reinfection were, however, complex and hard to interpret.

Bøe (6) published an extensive study of delayed-type skin hypersensitivity and its desensitization by staphylococci and staphylococcal products. In the hypersensitive rabbits, minimal doses of staphylococci caused large abscesses after intracutaneous injections of either living or Formalin-killed cultures. General systemic resistance was not tested, however.

Johanovsky (11) produced hypersensitivity in chinchilla rabbits by repeated intradermal and intravenous infections with small doses of staphylococci. Animals were selected after some weeks of treatment according to the intensity of skin reactions to diluted staphylococcal toxoid and vaccine given intradermally. Spleen cells or peritoneal exudate cells were transmitted intraperitoneally to normal recipient rabbits. The transfer of cells resulted in skin hypersensitivity of the delayed type to the recipients.

After the last reading for skin hypersensitivity, the animals were infected intravenously with  $2 \times 10^7$  virulent staphylococci. The bacteraemia and death rate in rabbits previously sensitized with cells of hypersensitive rabbits remained high in the course of infection, while the bacteraemia in control groups of normal rabbits, rabbits receiving normal cells or killed hypersensitive cells declined progressively.

Our finding that peritoneal macrophages from repeatedly infected rabbits are on the whole less staphylococidal than macrophages from normal animals thus confirms, at the cellular level, the important findings recorded by Johanovsky regarding staphylococcal infection in vivo.

Cluff, in reviewing the studies by himself and associates on the pathogenesis of staphylococcal

infection states (7): "Staphylococcal infections in human beings have an extraordinary capacity for recurrence and persistence. Frequently, recurrent infections are attributable to the same bacterial strains. Persons with recurrent infections usually have serum agglutinins and antitoxins, and display dermal hypersensitivity reactions to staphylococcal antigens."

In the accompanying paper (14), it is shown that the allergy which follows repeated infections with *S. aureus* does afford an essential basis from which appropriate specific elicitation can evoke increased resistance.

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