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Extent of Specific to Nonspecific Resistance in Mice: Parenteral Versus Aerosol Challenge

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Quantitative data were gathered concerning the extent of resistance induced in mice immunized by specific and nonspecific means and subsequently challenged both parenterally and by aerosol. Animals were immunized specifically by subcutaneous or intraperitoneal injection of Formalin-killed *Klebsiella pneumoniae* type I, which was also employed as a challenge organism. The intraperitoneal LD_{50} was 30 bacilli. Nonspecific resistance was induced by injection of a Boivin preparation of *Salmonella typhimurium* endotoxin. Nonspecific resistance was highest 24 hr after injection of 10 μg of endotoxin. At this time, more than half of the mice survived challenge with 10^2 but not with 10^3 LD_{50}. Specifically immunized mice were resistant to as much as 10^5 LD_{50}, depending upon the route of immunization. Potency ratios for parenteral challenge were: nonspecific to normal, 100; specific to normal, 10^4 to 10^5; specific to nonspecific, 10^2 to 10^3. Employing aerosol challenge, specific immunization protected in the LD_{100} range; nonspecifically immunized animals showed significant prolongation of survival time, but the 30-day mortality was similar to the control group.

The ability of gram-negative bacterial endotoxins to enhance resistance of experimental animals has been known and studied for many years. The mechanism of this endotoxin-induced nonspecific resistance is still unknown, although a number of hypotheses have been suggested (2, 5, 6, 12).

There are few reports concerning the comparative efficacy of specific and nonspecific resistance in a single experiment. Usually the studies are concerned with either specific or nonspecific resistance. The purpose of this study was to obtain quantitative data relating the degree of resistance induced in mice by parenteral introduction of gram-negative bacterial endotoxin to immunity induced by specific immunization. Experiments were designed to compare responses after both parenteral and aerosol challenge.

MATERIALS AND METHODS

Endotoxin preparation. The endotoxin used was lipopolysaccharide B *Salmonella typhimurium* (Difco). A stock solution was prepared by dissolving the endotoxin (400 μg/ml) in nonpyrogenic 1.0% gelatin in 0.9% saline (gel-saline). The stock solution was stored at 4 C.

Mice were injected with dilutions of the stock solution in 0.25-ml volumes; control animals received gel-saline by the same route.

Preparation of vaccine. Cultures of *Klebsiella pneumoniae* capsular type 1 were inoculated on Brain Heart Infusion (Difco) agar slants and incubated at 37 C for 24 hr. The organisms were harvested in gel-saline, and the opacity of the bacterial suspensions was adjusted to a MacFarland no. 3 tube (4); then the organisms were killed by the addition of Formalin to 0.5%. After an incubation period of 24 hr, 1.0 ml of the vaccine was spread on a nutrient agar plate and incubated. No growth was observed after 24 and 48 hr of incubation. The vaccine was stored at 4 C and used for as long as 1 month. Animals were injected with 0.25 ml of vaccine, with a period of 3 to 4 days between injections.

Bacterial challenge cultures. *K. pneumoniae* capsular type 1 cultures were obtained from the departmental stock culture collection and were maintained on stock culture medium (Difco) at room temperature. The organisms were inoculated onto Brain Heart Infusion agar slants, incubated at 37 C for 18 to 24 hr, and then harvested in gel-saline. The approximate number of organisms in the suspension was determined by obtaining turbidity readings on a Klett-Summerson photoelectric colorimeter with filter number 54 (approximate spectral range 500 to 570 nm). Direct plate counts were made to estimate more accurately the number of organisms per milliliter.

For parenteral challenge, dilutions of the organism suspensions were given intraperitoneally in 0.25 ml. Control animals received the same volume of gel-

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saline by the same route. The intraperitoneal LD_{50} for mice with this organism was determined by the method of Miller and Tainter (8) to be 30 $\pm$ 26 organisms. Animals were observed for a period of 5 days after parenteral challenge.

For aerosol challenge, 18- to 24-hr cultures of *K. pneumoniae* were harvested in gel-saline. It was found that a suspension of organisms, prepared such that a 1:10 dilution of the organisms used in the aerosol challenge had an optical density of 18 on the colorimeter, was sufficient to infect most of the control animals. Animals were observed after challenge until 3 days had elapsed without any deaths.

Aerosol apparatus. All aerosol challenges were carried out in an airtight plexiglass chamber measuring 3 by 2 by 2 feet (0.9 by 0.6 by 0.6 m). This chamber, modified from an apparatus previously described (9), contained an exhaust fan leading to a bacterial incinerator attached to the chamber. An Andersen sampler (model no. 0604) was attached to the top of the chamber by a short length of Tygon plastic hose [1 inch (2.54 cm) in diameter] which was clamped off when the sampler was not in operation. A fan was placed inside the chamber to keep the air circulating while the aerosolizer was running. The bacterial suspension was aerosolized by means of an aerosol generator (model 200A, Schoeffel Instrument Co.). The machine aerosolized the suspension at a rate of 1 ml per 5 min or 12 ml per hr. The animal cages were placed in the chamber at the end opposite the aerosolizer and fan. The animals were exposed to the aerosol for 1 hr. A 15-sec air sample was taken with the Andersen sampler (1) after 5 min of aerosolization, and plate counts were subsequently made after 24 hr of incubation. By using the described bacterial challenge suspension in the aerosolizer, the sixth Andersen plate (particles less than 1 μm) showed from 10 to 42 particles of bacterial suspension in 0.25 ft^3 of air. Plates 4 and 5 (1 to 4 μm) showed 832 to 2,127 particles per 0.25 ft^3 of air. Less than 10% of the larger particles reach the alveolar bed (10). Sterile air was passed through the chamber for 30 min after aerosol challenge, the mice were removed, and the aerosol chamber was then sterilized with ethylene oxide gas.

Animals. Adult albino mice (*Mus musculus*) of both sexes, obtained from a local source, were used throughout. Animals were allowed free access to food and water before and during the course of the experiments.

RESULTS

Time of administration of endotoxin. The results shown in Table 1 illustrate that nonspecific resistance induced by endotoxin was maximal at 24 hr and rapidly diminished thereafter. This observation is similar to that reported originally by Rowley (11). No significant protection of the challenged mice was evident if the challenge was made 48 hr after endotoxin treatment. In all of the following experiments, challenge with *K. pneumoniae* (intraperitoneal or aerosol) was made from 23 to 25 hr after administration of endotoxin.

TABLE 1. *Duration of enhanced resistance after intraperitoneal (IP) administration of 10 μg of endotoxin*

Time between injection of endotoxin and IP challenge of 10 LD_{50}	No. of mice dead/total at the day indicated after challenge				
	1	2	3	4	5
1 day	0/10	1/10	2/10	3/10	3/10
2 days	0/10	3/10	7/10	7/10	7/10
3 days	0/10	6/10	8/10	8/10	8/10
4 days	1/10	4/10	7/10	7/10	7/10
5 days	2/10	4/10	7/10	8/10	8/10
24 hr[a]	5/10	9/10	9/10	9/10	9/10

[a] Control, gel-saline administered IP 24 hr before challenge.

TABLE 2. *Nonspecific immunization against Klebsiella pneumoniae in mice*[a]

Challenge (LD_{50})	Amt (μg) of endotoxin injected				
	Control (gel-saline SC)[b]	0.1	1.0	10	100
1	4/10[c]	6/10	0/9	1/10	1/10
10	10/10	8/10	6/10	2/10	0/10
10^2	9/10	9/10	3/10	3/10	3/10
10^3	10/10	10/10	10/10	10/10	9/10
10^4	10/10	10/10	10/10	10/10	9/9

[a] Endotoxin injected subcutaneously 24 hr before intraperitoneal challenge.
[b] SC, subcutaneously.
[c] Number dead/total 5 days after challenge.

Effect of specific and nonspecific vaccination followed by parenteral challenge. The ability of endotoxin to influence the resistance of mice to intraperitoneal challenge with *K. pneumoniae* is evident from the data shown in Table 2. Endotoxin was administered subcutaneously in the nuchal region 24 hr before challenge. The data in Table 2 show that 0.1 μg of endotoxin gave no significant protection, whereas 1, 10, and 100 μg gave approximately equal protection. It is noteworthy that the nonspecific protection was not due to any preinduced inflammation reaction at the site of challenge. It can be seen that mice were protected against challenge doses of as much as 10^2 LD_{50} but that no protection was given against challenge doses of 10^3 LD_{50}.

Table 3 shows the results of a similar experiment. In this case, endotoxin was used as before but in one concentration only, that is 10 μg given intraperitoneally. The 5-day mortality ratios suggest that there was significant protection against 10^2 LD_{50} but not against 10^3 LD_{50}.

TABLE 3. *Nonspecific immunization against Klebsiella pneumoniae in mice*[a]

Challenge (LD_{50})	No. dead/total on day indicated after challenge				
	1	2	3	4	5
Control[b]	0/10	6/10	7/10	8/10	9/10
10	0/10	0/10	1/10	1/10	1/10
10^2	0/10	1/10	2/10	2/10	4/10
10^3	1/10	5/10	8/10	9/10	9/10

[a] Endotoxin (10 μg) injected intraperitoneally 24 hr before intraperitoneal challenge.

[b] Challenge of 10 LD_{50} but gel-saline intraperitoneally instead of endotoxin.

TABLE 4. *Specific immunization against Klebsiella pneumoniae in mice*[a]

Challenge (LD_{50})	No. of immunizations before challenge				
	Control (gel-saline SC)[b]	1×	2×	3×	4×
10^3	9/10[c]	6/10	4/9	3/9	3/9
10^4		10/10	8/10	2/9	9/10
10^5		10/10	10/10	9/10	9/10
10^6		9/9	9/9	9/9	9/9

[a] Vaccinated subcutaneously, challenged intraperitoneally.

[b] SC, subcutaneously.

[c] Number dead/total 5 days after challenge.

Therefore, under these experimental conditions, there was no difference in the protection induced when the endotoxin was given subcutaneously or intraperitoneally.

Data on protection induced by specific immunization are given in Table 4. Four groups of 40 mice each were immunized subcutaneously with *Klebsiella* vaccine. The first group received four immunizing injections, the second group received three, the third group received two, and the fourth group received one. Four days after the last immunization, mice were challenged with the dose amounts noted in Table 4. Animals receiving three and four vaccine injections were obviously made ill by this procedure. Fur was ruffled and skin lesions were present. The evidence in the table suggests that specifically immunized animals could be protected against 10^4 LD_{50} but not against 10^5 LD_{50}.

Table 5 shows the results of specific immunization with the same vaccine. In this case, however, the vaccine was injected intraperitoneally. The animals received four vaccinating injections. Mice, when vaccinated by this route, were protected against at least 10^5 LD_{50}. The animals reacted far less to vaccine administered intraperitoneally than they did to the subcutaneous immunization.

TABLE 5. *Specific immunization against Klebsiella pneumoniae in mice*[a]

Challenge (LD_{50})	No. dead/total on the indicated day after challenge				
	1	2	3	4	5
Control[b] (gel-saline IP)	10/10				
10^3	1/10	1/10	1/10	1/10	1/10
10^4	0/10	0/10	1/10	1/10	1/10
10^5	1/10	1/10	2/10	2/10	2/10

[a] Vaccinated four times intraperitoneally, challenged intraperitoneally.

[b] Challenge of 10^3 LD_{50}. IP, intraperitoneally.

TABLE 6. *Specific and nonspecific immunization against Klebsiella pneumoniae via aerosol challenge*

Group	Mortality ratio after	
	7 days	16 days
Control (gel-saline IP)[a]	19/20	20/20
Nonspecific (10 μg of endotoxin IP)	6/20	18/20
Specific (4× IP)	7/20	10/20

[a] IP, intraperitoneally.

Effect of specific and nonspecific vaccination followed by aerosol challenge. Mortality ratios after aerosol challenge in the LD_{100} range are shown in Table 6. The specifically immunized mice were significantly protected ($P = <0.01$), whereas the nonspecifically immunized mice showed significant prolongation of survival time ($P = <0.01$ at 7 days) but no differences in mortality. Table 7 shows results of three other separate experiments in which the mice were challenged via the aerosol route. Table 8 summarizes the results of these four experiments.

At the conclusion of the fourth aerosol experiment, cultures were made of the lungs of the surviving animals. The challenge organism, *K. pneumoniae*, was recovered from only one mouse. On all cultures taken from mice in the LD_{100} challenge group, the organism could only be recovered from the lungs of acutely ill mice. These data indicate that the mice do not harbor the organism in a chronic disease state.

DISCUSSION

Statistical analysis of the data obtained when mice were nonspecifically immunized either sub-

TABLE 7. *Specific and nonspecific immunization against Klebsiella pneumoniae via aerosol challenge*

Groups	Expt 1			Expt 2			Expt 3		
	4 days	7 days	11 days	4 days	7 days	11 days	4 days	7 days	11 days
Control (gel-saline IP)	5/10[a]	9/10	10/10	3/10	4/10	6/10	0/10	3/10	4/10
Nonspecific (10 μg of endotoxin IP)	3/10	7/10	8/10	3/10	4/10	5/10	0/10	1/10	3/10
Specific (4× vaccine IP)	1/10	5/10	7/10	2/10	2/10	2/10	0/10	1/10	2/10

[a] Number dead/total at day indicated after challenge.

TABLE 8. *Specific and nonspecific immunization against Klebsiella pneumoniae via aerosol challenge*[a]

Groups	No. dead/total at indicated day after challenge	
	7 days	11–16 days
Control (gel-saline IP)	25/50	40/50
Nonspecific (10 μg of endotoxin IP)	18/50	34/50
Specific (4× vaccine IP)	15/50	21/50

[a] Summary of experiments 1, 2, and 3.

cutaneously or intraperitoneally shows that 0.1 μg of endotoxin gave no significant protection to mice challenged intraperitoneally with *K. pneumoniae*. Significant protection at the 1% level of probability using minimum contrast tables (7) is seen with 1, 10, and 100 μg of endotoxin against challenge with 1, 10, and 10^2 LD_{50}. Also, no difference in survival rate exists between test and control animals in groups challenged with 10^3 and 10^4 LD_{50}. The potency ratio (3) is then 10^2 for mice nonspecifically immunized and challenged parenterally in the described manner.

In contrast, the experiments concerning specific immunization followed by parenteral challenge show significant protection at the 1% level, using minimum contrast tables, against challenge with 10^3 and 10^4 LD_{50}. When mice were vaccinated subcutaneously, no significant protection was conferred against challenge with 10^5 or 10^6 LD_{50}. When vaccine was injected intraperitoneally, animals were protected against 10^5 LD_{50}. In this defined situation, the potency ratio for specific immunization subcutaneously is 10^4 and for specific immunization intraperitoneally is 10^5.

The relative potency of the two described methods of immunization against parenteral challenge is obtained as the ratio of potency of specific to nonspecific immunization, $10^5/10^2$, which is 10^3. This value indicates that when mice are immunized intraperitoneally with a specific *K. pneumoniae* vaccine and are subsequently challenged parenterally with *K. pneumoniae*, they show 1,000 times the protection induced by immunization with endotoxin as described.

When mice were challenged by aerosol, the LD_0 and LD_{100} doses of *K. pneumoniae* seemed to be very close. This fact made it difficult to carry out experiments with an adequate number of animals. To get significant differences between specifically and nonspecifically immunized and nonspecifically immunized and control groups of mice, the results given in Table 8 would have to be obtained with more than 500 animals instead of 50. However, mice specifically immunized to the challenge agent were significantly protected as compared to the control group ($P = <0.01$), and in every experiment the mortality of the nonspecifically immunized animals was greater than the specifically immunized groups but less than the control groups.

When more than the described number of organisms were administered (LD_{100} range), the mortality ratios of the three groups of mice were not significantly different at the conclusion of the experiment. However, the control mice started dying sooner than both specifically and nonspecifically immunized animals, and the specifically immunized animals survived longer than the nonspecifically immunized animals. In terms of mortality, there is no difference noted, but the mortality time is affected by immunization.

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