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Alveolar macrophage bactericidal activity   Maturation phagocytic uptake

Maturation of the Rabbit Alveolar Macrophage during Animal Development III. Phagocytic and Bactericidal Functions

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Summary

Phagocytic and bactericidal function of rabbit alveolar macrophages (AMs) lavaged from animals during the course of postnatal maturation was studied. *Staphylococcus aureus* and a temperature-sensitive mutant of *Escherichia coli*, which could not replicate at 37° during the functional assays, were employed as test bacteria. Assays of the phagocytic capacity of AMs from rabbits of various age groups revealed no significant differences either in the percentage of AMs which took up bacteria (79–90%) or in the number of bacteria taken up per AM (Table 1). In contrast, bactericidal activity of AMs was found to increase with increasing animal age. No bactericidal activity was detected in AMs from newborn animals (Figs. 1 and 2), whereas AMs from 7-day-old animals exhibited at least a bacteristatic activity against *S. aureus* (Fig. 1) and AMs from 28-day-old rabbits showed marked bactericidal activity, essentially the same as that of AMs from adult rabbits. Adult AMs killed 75% of the *S. aureus* and 60% of the *E. coli* within 120 min (Figs. 1 and 2).

Speculation

The development of bactericidal activity in AMs during the postnatal period correlates with their previously reported morphologic and biochemical maturation. This developmental pattern of bactericidal activity may indicate that the mechanisms responsible for bacterial killing may not be fully developed at birth but develop during extrauterine life. Alternatively, the large quantities of phagocytized surfactant-like material known to be present in the AMs in the early postnatal period may inhibit their bactericidal activity.

We have recently reported ultrastructural and biochemical studies on the rabbit alveolar macrophage (AM) during animal development (23, 32). Significant ultrastructural changes were observed during the first postnatal month of life, including progressive development of the rough endoplasmic reticulum and Golgi complex and marked increases in the cellular content of lysosomes and mitochondria (32). Biochemical changes observed during the same period included increases in the cellular content of several lysosomal enzymes and oxidative enzymes (23). The present investigation was undertaken to examine the phagocytic and bactericidal capabilities of AMs during animal development and then to correlate these functional parameters with the previously reported ultrastructural and biochemical findings.

MATERIALS AND METHODS

ANIMALS

AMs were obtained from New Zealand white rabbits of both sexes (B and H Rabbitry, Rockville, Md.). Groups of four to six rabbits of each of the following ages were employed: 6–20 hr after birth (1 day); 7 and 28 days after birth; 90 or more days after birth (considered adult).

ISOLATION OF MACROPHAGES

AMs were obtained from animals by tracheobronchial lavage as previously described (32). After the lavage procedure, cell suspensions were centrifuged at 150 × *g* for 5–10 min at 4°, then washed twice with sterile Krebs-Ringer phosphate buffer, pH 7.2, containing 200 mg% glucose and 1.5 mM Ca^{++} (KRP) at 4°, and used immediately. In order to obtain a sufficient quantity of cells, suspensions from littermates of 1-day- and 7-day-old animals were pooled.

PREPARATION OF BACTERIAL SUSPENSIONS

Staphylococcus aureus var Copenhagen (*S. aureus*) and *Escherichia coli* strain Easter (*E. coli*), obtained from Dr. J. B. Robbins (National Institute of Child Health and Human Development, Bethesda, Md.), were used as test organisms for assays of phagocytic uptake and bactericidal activity. Before use, the *E. coli* was mutagenized chemically as described elsewhere (16), and a mutant was selected whose replication was reversibly inhibited at 37°, but which grew normally at 25°. This mutant could be employed in the phagocytic and bactericidal assays to be described (at 37°) without the complication of rapid bacterial replication during the course of the assays.

Bacterial suspensions of these two organisms were prepared for use in assays from overnight broth cultures by washing the cultures twice in KRP and resuspending them at an optical density which yielded 2–4 × 10^8 colony-forming units/ml. These suspensions were kept at 4° until use.

PREPARATION OF NORMAL RABBIT SERUM (NRS)

A single preparation of NRS was obtained by pooling fresh serum from 10 adult rabbits. This preparation was divided into aliquots, stored at −70°, and used in all phagocytic and bactericidal assays.

ASSAY OF PHAGOCYTIC UPTAKE

Monolayers of AMs were prepared from lavage suspensions as described elsewhere (32). These monolayers were incubated with 2–4 × 10^7 test bacteria suspended in 4% NRS for 30 min at 37° with gentle agitation. After the incubation period, monolayers were washed thoroughly with 0.5% NaCl, air-dried, methanol-fixed, and stained with 3% Giemsa solution. The percentage of AMs which had taken up bacteria, as well as the number of bacteria associated with each AM, were determined by light microscopy on duplicate slides by examining at least 100 cells on each slide.

Table 1. *Phagocytic uptake of bacteria by alveolar macrophages lavaged from animals of different ages*[1]

	1 day old		7 day old		28 day old		>90 day old	
	S. aureus	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*	*E. coli*
% Macrophages with associated bacteria	90 ± 5[2]	79 ± 5	85 ± 2	82 ± 5	88 ± 3	81 ± 3	89 ± 2	88 ± 2
No. of bacteria/macrophage	16 ± 3[3]	8 ± 5	15 ± 5	5 ± 3	11 ± 1	6 ± 5	14 ± 5	8 ± 4

[1] Five to nine experiments were performed in duplicate for each age group.
[2] Mean ± SEM.
[3] Mean ± SD.

ASSAY OF BACTERICIDAL ACTIVITY

The method of Quie (25) was modified as follows to permit assay of the small number of AMs obtainable from the younger animals. In siliconized, sterile BEEM capsules (Ernest F. Fullam Inc., P.O. 444, Schenectady, N. Y.), 0.2 ml of the washed macrophage suspension, containing 4×10^5 AMs, was incubated with an equal number and volume of the test bacteria in the presence of 4% NRS. Control incubations were performed in identical preparations without AMs. All preparations were carried out in triplicate and incubated at 37° on a Fisher Roto-Rack (Fischer Scientific) at 10 rpm. Aliquots were removed from each preparation at 0, 60, and 120 min, and serially diluted (1:100 and 1:10) with sterile water to lyse the AMs, using a 15-min incubation in each dilution. This procedure was found to result in complete disruption of the macrophages without a decrease in colony-forming units (CFU) of bacteria. CFU were determined on the final dilution of each aliquot, and results were expressed as a percentage of the CFU present in the zero time aliquot. At the end of the incubation period, additional aliquots were examined to determine the viability of the AMs and to check for any loss in the number of suspended AMs which might have resulted from lysis or adherence. No loss of viability or decline in number of suspended AMs was detected in any preparations.

In a separate set of experiments, the effect of alveolar lining material (ALM) on the phagocytic and bactericidal functions of AMs was tested. This material has been reported to enhance bactericidal activity in rat AMs (19). ALM was prepared from the pooled lavagates obtained from two normal adult rabbits. These lavagates were centrifuged at 200 × *g* for 15 min at 4° to remove the cells, and the supernatants were pooled and centrifuged at 40,000 × *g* for 20 min at 4°. The resulting pellet was resuspended in 6 ml KRP and is referred to as ALM. Test bacteria were suspended in either ALM or KRP and incubated for 60 min at 37°. Phagocytic and bactericidal assays were then performed with AMs from 7-day-old and adult animals as described above, using these suspensions of test bacteria, both in the presence and absence of NRS.

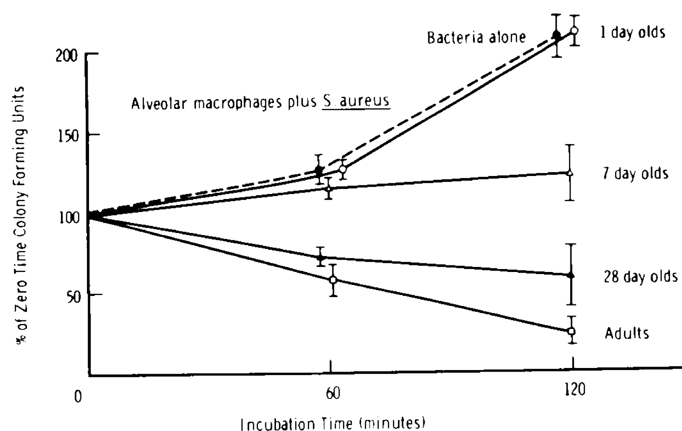

Fig. 1. Bactericidal activity of alveolar macrophages lavaged from rabbits of different ages using *S. aureus* as test bacterium. Five or more experiments were performed in triplicate for each age group and the values are expressed as percentage of zero time colony-forming units present (mean ± SEM).

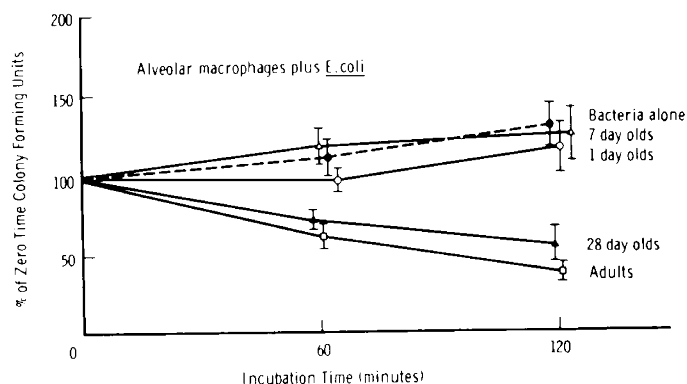

Fig. 2. Bactericidal activity of alveolar macrophages lavaged from rabbits of different ages using *E. coli* as test bacterium. Five or more experiments were performed in triplicate for each age group and the values are expressed as percentage of zero time colony-forming units present (mean ± SEM).

RESULTS

The results of assays of phagocytic uptake of test bacteria by AMs lavaged from animals of different ages appear in Table 1. It can be seen that 85–90% of the AMs lavaged from animals of all ages took up an average of 11–16 *S. aureus* each. Similarly, 79–88% of AMs took up an average of 5–8 *E. coli* each. No significant difference in either the percentage of AMs which took up bacteria or in the number of bacteria taken up per AM was found in comparing the various age groups.

The results of assays of the bactericidal activity of AMs with test bacteria are shown in Figures 1 and 2. It may be seen that AMs from 1-day-old rabbits failed to reduce the numbers of either *S. aureus* (Fig. 1) or *E. coli* (Fig. 2) which were present at zero time and, in fact, CFU of these bacteria increased by 100% and 30%, respectively, during the incubation periods. Similar increases in CFU were observed in control preparations (which lacked AMs). AMs from 7-day-old rabbits also showed no significant ability to reduce numbers of either test bacterium within the 120-min incubation period employed, but these AMs at least partially prevented the increase in *S. aureus* observed in control preparations. However, these AMs from 7-day-old animals had no measurable effect on the number of (nonreplicating) *E. coli* (Fig. 2). AMs from 28-day-old rabbits showed a significant bactericidal activity against both test bacteria, eliminating approximately 40% of the initial innoculum of either organism within 120 min (Figs. 1 and 2). AMs from adult animals showed still greater bactericidal activity, eliminating 75% of the *S. aureus* (Fig. 1) and 60% of the *E. coli* (Fig. 2) within 120 min.

The results of phagocytic and bactericidal assays using test bacteria which were preincubated with ALM were not significantly different from those reported in Table 1 and Figures 1 and 2.

DISCUSSION

The results of the present study indicate that lavageable AMs from rabbits of the various ages tested were highly phagocytic

when assayed with either *S. aureus* or *E. coli*, and that the degree of phagocytic uptake was similar in AMs from all age groups. In contrast to phagocytic activity, bactericidal activity in AMs was found to increase with increasing animal age. Bactericidal activity was not detected in AMs from newborn animals; however, AMs from 7-day-old animals at least partially prevented the increase in CFU of *S. aureus* during the assay period. This might imply the presence of a bacteristatic activity, detectable only against rapidly replicating bacteria such as *S. aureus*. Alternatively, this apparent bacteristatic activity could result from bactericidal mechanisms which are either incompletely developed in AMs at this time or are present in only a portion of the AM population. AMs from 28-day-old rabbits showed the first significant bactericidal activity against both test bacteria and this activity was similar to that found in AMs from adult animals. Employing a microscopic microbicidal technique using *Candida albicans* as a test organism, a similar pattern of microbicidal activity was observed with the AM preparations from all age groups (33).

Two possible explanations for the observed pattern of development of bactericidal activity during animal maturation seem worthy of consideration. The first is that the bactericidal mechanisms of the AM may be fully developed at birth but may be inhibited at this time by the large quantities of phagocytized surfactant-related material present in these cells during the early postnatal period (23, 32). Such a large phagocytic uptake in macrophages has been found to correlate with a reduction in observable lysosomes (31, 32), and has also been reported to result in the depletion of cellular energy reserves (24), effects which may interfere with normal bactericidal activity. Furthermore, macrophages loaded with erythrocytes show a decreased ability to destroy bacteria (13). And in fact, AMs which have ingested proteinaceous alveolar fluid, which is morphologically similar to the surfactant-related material present in the newborn AMs (32), exhibit substantially decreased microbicidal activity (14). A second possible explanation, not exclusive of the first, for the observed developmental pattern of bactericidal activity is that the mechanisms which are responsible for bacterial killing by AMs may not be fully developed at birth but develop subsequently. This explanation would correlate with *in vivo* studies of peritoneal and tissue macrophages which show a prenatal development of phagocytic function and a similar postnatal development of bactericidal mechanisms (15, 17, 26–28). Moreover, the postnatal development of bactericidal mechanisms in macrophages is also suggested by the reported ability of transferred adult peritoneal macrophages to protect newborn animals against otherwise lethal infections (7).

Although the precise mechanisms involved in the bactericidal function of AMs remain unsettled (1-4, 6, 8-12, 18, 21, 30), some of the morphologic changes which occur with animal development (32) (including increased development of the Golgi apparatus, RER, lysosomes, and mitochondria) correlate with the development of this function. Moreover, the increases in cellular content of several enzymes which occur with animal development (23), particularly lysozyme and the enzymes of the hexosemonophosphate shunt, important in bactericidal function of the other phagocytes (18), also correlate with the development of bactericidal activity in AMs. Similar changes in hexosemonophosphate shunt enzymes have been shown to occur in human neutrophils during development (5). These correlations suggest that the morphological changes and the increases in cellular enzyme content may in part be related to the maturation of bactericidal activity in AMs and are similar to those reported in BCG-stimulated AMs which also have increased bactericidal activity (9, 20, 22, 29). The possible implications of these findings in AMs after birth may be important in explaining the high frequency of respiratory infections characteristic of the newborn animal.

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Partially Restricted Antitoxins of Tetanus and Diphtheria in Man

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Summary

Antibodies of restricted specificity have been identified in the human in response to certain antigens. The present study analyzed tetanus and diphtheria antitoxins isolated from selected human sera and suggested a restricted reponse in antibody production to each of these antigens. Purified antibodies from eight serum specimens with elevated hemagglutination titers to tetanus and four to diphtheria yielded only IgG proteins in concentrations of 160–500 μg/ml. Although some of the tetanus specimens were derived from cord sera and tetanus immunoglobulin, none of the total group had antibodies of the IgA and IgM classes. Utilizing immunoelectrophoresis against heavy chain subclasses, genetic markers, and κ and λ quantitations, a predeliction for the κ IgG_1 subclass was established for both tetanus and diphtheria antibodies. The λ light chains were present in diminished quantities, IgG_2 heavy chains were absent, and the IgG_3 and IgG_4 chains were variably identified.

Speculation

The elucidation of those antigens which result in restricted antibody formation has apparent clinical significance, in view of the fact that some individuals possess selective defects in the production of certain IgG subclasses.

The light and heavy chain differences cause human immunoglobulins of each of the classes to exist in several forms, in accordance with variations in the way in which these chains combine. Since IgG heavy chains, as an example, occur in four subclasses (IgG_1, IgG_2, IgG_3, and IgG_4) and the light chains in two types (κ and λ), eight different IgG molecules are possible by varying combinations of light and heavy chains. Normally, the IgG_1 subclass constitutes about 66% of total IgG, 23% IgG_2, 7% IgG_3, and 4% IgG_4. When combined with the IgG_1 chain, the light chain κ:λ ratio is about 2:1.

Although most antibodies produced in the human appear to be heterogeneous, the response to certain antigens may result in antibody immunoglobulin which is relatively or even completely restricted. Rh antibody, as an example, is primarily an IgG_1 immunoglobulin with some IgG_3 and IgG_4 but no IgG_2 molecules (5, 10), whereas bacterial polysaccharide antigens elicit predominantly IgG_2 production (7). Antibodies against factor VIII are formed preferentially within the IgG_4 subclass (1). Levan provokes a highly restricted manufacture of κ IgG_2 globulins, approaching the homogeneity of a myeloma protein (17).

Utilizing available immunologic techniques, criteria have been proposed for establishing antibody specificity (3, 16). Employing several of these criteria, the results of this investigation suggest that the human responds with partial antibody restriction to the toxins of *Clostridium tetani* and *Corynebacterium diphtheria*.

MATERIALS AND METHODS

Eight specimens containing tetanus antibodies were derived from three sources: five were samples of newborn cord sera, two were taken from human antitetanus immunoglobulin pools, and one was serum of a 5-year-old burn patient taken several weeks after a tetanus toxoid booster immunization. Four sera containing diphtheria antibodies were from children, aged 7 months to 11 years, with recent clinical diphtheria. Specimens were obtained within a period not exceeding 6 months from the time of active disease. Antibody titers to tetanus and diphtheria in sera and in purified antibody preparations were measured by a hemagglutination technique using the toxoids of either antigen coupled to human type O Rh⁻ red blood cells by the chromic chloride method (2).

Purified antibody preparations were prepared in the following manner. Tetanus or diphtheria toxoid (Wyeth, aluminum-precipitated toxoid containing 0.01 mg protein N/ml for tetanus and 0.004 mg protein N/ml for diphtheria) (3–4 ml) was incubated with 1 ml serum for 1 hr at 37°, then refrigerated overnight. The precipitate was washed 12 times. The supernatant and early washes were discarded after being tested for specific antibody activity. The precipitate was then incubated with acid-glycine buffer, pH 3.0 (1 ml), overnight at room temperature. The supernatant was dialyzed against normal saline for 1 hr and the residual material discarded. The dialysate (about 1 ml in quantity), representing a purified antibody preparation, was used for subsequent analyses.