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Microbicidal Activity of Peroxidaseless Chicken Heterophile Leukocytes

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Polymorphonuclear leukocytes (PMN) of the domestic chicken lack myeloperoxidase and, therefore, may be useful for studies of myeloperoxidase-independent antimicrobial mechanisms. Before such studies were undertaken, it was important to investigate the antimicrobial capacity of these cells against species of opportunistic pathogens that cause infection in humans with defective PMN function. In vitro, chicken PMN, like normal human PMN, readily phagocytized and killed *Staphylococcus albus*. They also killed *Serratia marcescens*, *Escherichia coli*, and *Candida albicans*. Cytochemical methods confirmed the absence of myeloperoxidase from chicken PMN.

Myeloperoxidase (MPO) has attracted renewed attention throughout the last decade and is now believed to be of major importance in the microbicidal function of polymorphonuclear leukocytes (PMN). The system responsible for the intracellular killing of ingested organisms is thought to consist of MPO, H_2O_2, and oxidizable cofactors. This hypothesis presently is supported by demonstrations of the antimicrobial effects of MPO, H_2O_2, and oxidizable cofactors (7, 13), observations on failure of candidicidal function in PMN from a patient deficient in MPO (9, 10), and failure of bactericidal function in PMN, apparently unable to generate H_2O_2, from patients with chronic granulomatous disease (6, 14).

Under some circumstances, however, PMN may be deficient in MPO without any increase in susceptibility to infection being observed in the patients. Two of three patients found to lack MPO in their PMN appear to be healthy (4, 17), and the third one suffered from candidiasis (and diabetes) but not from recurrent bacterial infections (10). It would seem advantageous, therefore, to investigate antimicrobial mechanisms independent of MPO in a model based on reliable supplies of PMN that naturally lack MPO.

To study the involvement of antimicrobial systems independent of MPO in PMN, we took advantage of the phylogenetic difference of avian PMN. We subjected various microorganisms to the action of chicken heterophile leukocytes which completely lacked MPO (15).

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MATERIALS AND METHODS

Chickens. Male, white Leghorn chickens, each weighing about 1.5 kg, were obtained from a local breeding farm. They were kept under laboratory conditions and fed with antibiotic-free food.

Cells. The chicken PMN were obtained from peritoneal exudates 12 to 14 hr after an injection of 1% glycogen-saline, care being taken to avoid the peritoneal air sacs. The cells were washed twice in Hanks balanced salt solution (BSS), and the PMN were then purified by low-speed centrifugation (60 × *g*, 15 min). This centrifugation left the major portion of the thrombocytes and lymphocytes in the supernatant fluid and yielded a practically aggregate-free pellet, which consisted of more than 80% PMN, about 15% macrophages, and only 5% thrombocytes and lymphocytes. Smears stained by the method of Graham-Knoll (16), a peroxidase stain followed by Romanowsky counterstain, revealed 0 to 6% peroxidase-positive eosinophils and 94 to 100% peroxidase-negative heterophils. Chicken heterophils or PMN possess large, oval, eosinophilic granules which are peroxidase-negative. Chicken eosinophils, unlike the PMN, possess spherical, peroxidase-positive granules, which are eosinophilic with Romanowsky stains. Homogenized PMN suspensions, unless they included eosinophils, contained no detectable peroxidase activity when analyzed by the method of Lück (11).

Microorganisms. The microorganisms subjected to the killing action of chicken PMN were *Escherichia coli* K-12, *Serratia marcescens*, *Staphylococcus albus*, and *Candida albicans*. We selected these microorganisms because all of them have only limited pathogenic potential in humans, but *E. coli* and *S. marcescens* have been isolated frequently from children suffering from chronic granulomatous disease of childhood (5). Moreover, *C. albicans* is reported to resist human PMN lacking MPO (10).

Bactericidal assay. The method for the bactericidal assays was adapted from that of Quie et al. (14). PMN and bacteria were mixed in 10% normal chicken serum-BSS, incubated for 30 min at 37 C, washed three times to remove unphagocytized bacteria, and returned to incubation. Samples were taken from the reaction mixtures at various times, the PMN being lysed in sterile distilled water to release remaining viable bacteria. Colony counts were made from standard nutrient agar pour plates with the results expressed as the number of remaining bacteria per milliliter of reaction mixture. The original supernatant fluid and the three wash supernatant fluids were also counted for the number of viable bacteria. The difference between the original number of bacteria in the reaction mixture and the sum of the bacteria in the four supernatant fluids was taken to represent the phagocytic uptake. This figure was plotted at time zero, as if all phagocytosis had been instantaneous, in an effort to represent intracellular killing. The experiment with human PMN, shown for comparison, was performed by the same method with leukocytes obtained from freshly drawn, heparinized peripheral blood by dextran sedimentation.

Candidacidal assay. For comparison we estimated the candidacidal potency of chicken PMN by using the method described by Lehrer (9). In this method, *Candida* in yeast phase are incubated with PMN and autologous serum for 1 hr. After incubation, the PMN are lysed with deoxycholate, and methylene blue is added. An estimation of the killed and vital *Candida* is made by determining the ratio of killed (blue) to intact (unstained) organisms. We believe that this method underestimates the actual number of killed *Candida*, as we found a large portion of the organisms so completely digested in 60 min that staining and detection of such cells was difficult.

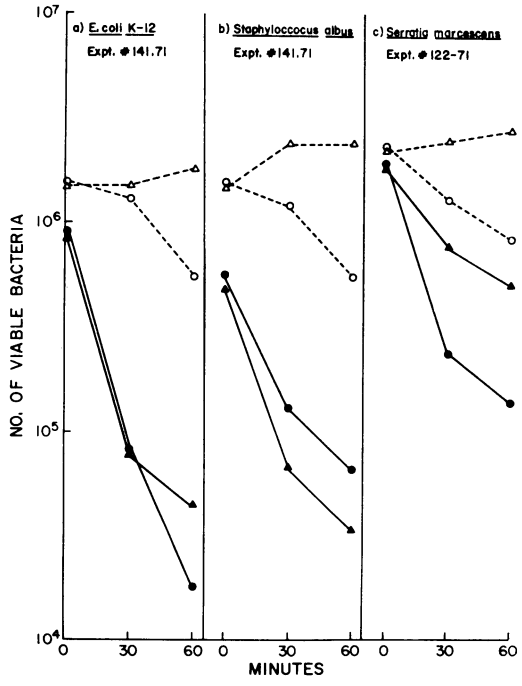

FIG. 1. *Killing of different bacteria by chicken polymorphonuclear leukocytes (PMN) or serum, or both. Symbols: (△---△), bacteria incubated with heat-inactivated chicken serum; (○---○), bacteria incubated with normal chicken serum; (▲—▲), bacteria incubated with PMN and heat-inactivated chicken serum; (●—●), bacteria incubated with PMN and normal chicken serum. The killing graphs for the PMN-bacteria-serum mixtures are plotted as follows: time zero, bacteria phagocytized by PMN = bacteria in original reaction mixture minus bacteria not sedimentable with PMN at 30 min. Bacteria in cells at 30 and 60 min = viable bacteria recovered from cells at 30 and 60 min. Where serum and bacteria alone were used, the numbers of viable bacteria in the suspending medium are plotted.*

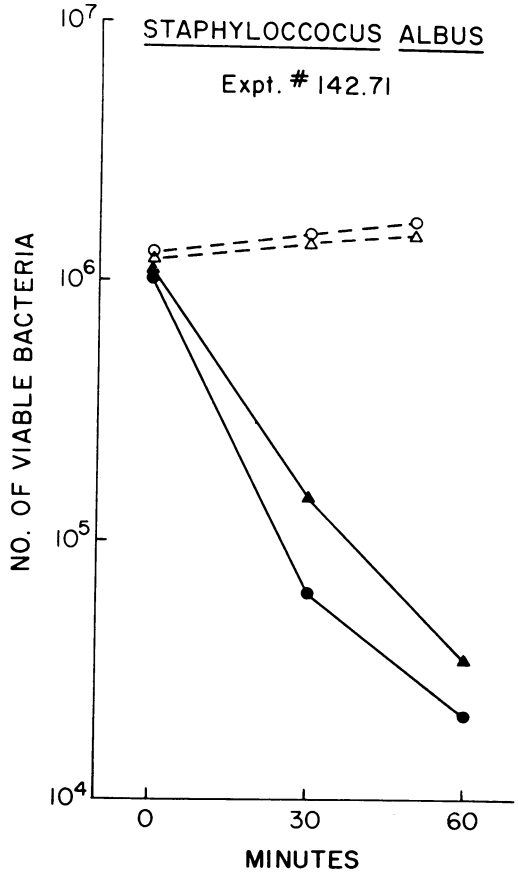

FIG. 2. *Killing of Staphylococcus albus by human polymorphonuclear leukocytes (PMN). Symbols: (△---△), bacteria incubated with standard control serum (not heat-inactivated); (○---○), bacteria incubated with patient's serum; (▲—▲), bacteria incubated with PMN and control serum; (●—●), bacteria incubated with PMN and patient's serum. The killing graphs are plotted exactly as in Fig. 1.*

TABLE 1. *Uptake and killing of bacteria by chicken polymorphonuclear leukocytes (PMN) in normal or heat-inactivated chicken serum*

Experimental conditions[a]	Serum	Bacteria					
		Escherichia coli K-12		*Serratia marcescens*		*Staphylococcus albus*	
		Killing[b]		Killing		Killing	
Serum alone							
After 15 min	Inactive	0		0		0	
	Normal	9		58		0	
After 30 min	Inactive	3.3 ± 7.4		0		0	
	Normal	37.5 ± 16.0		47.7 ± 7.6		24.3 ± 5.9	
		Uptake[c]	Killing[d]	Uptake	Killing	Uptake	Killing
Serum + PMN							
After 15 min	Inactive	8	0	69	80	32	87
	Normal	41	78	61	87	45	89
After 30 min	Inactive	77.0 ± 12.2	95.0 ± 3.3	77.3 ± 2.5	66.0± 7.0	26.7 ± 14.5	69.3 ± 16.5
	Normal	87.8 ± 15.0	96.6 ± 3.4	83.0 ± 7.9	82.0±13.2	62.0 ± 20.7	84.3 ± 6.7

[a] The 15-min values are from single experiments; the 30-min values represent the mean and standard deviation of four experiments.
[b] Per cent decrease of viable bacteria killed by serum alone.
[c] Per cent of bacterial inoculum phagocytized by PMN.
[d] Per cent of total ingested bacteria killed by PMN.

RESULTS

The results of our experiments are summarized in Table 1 and in Fig. 1, 2, and 3. They show that chicken PMN are capable of ingesting and killing gram-negative and gram-positive bacteria as well as yeasts. In the bactericidal assays, the uptake of bacteria is incomplete after 15 min but is nearly complete after 30 min (Table 1). These killing figures are slightly lower than other reported results obtained with normal human blood PMN (14). Figure 2 is given strictly for comparison and shows our results from a typical bactericidal assay using human PMN against *S. albus* in exactly the same procedure used with the avian PMN. The killing is comparable to that seen with chicken PMN, the only difference being that human PMN appear to phagocytize more of the initial inoculum than do chicken PMN (uptake by human PMN, ~95%; by avian PMN, ~60%). However, with the chicken PMN practically all of the ingested bacteria are killed—more than 80% in the first 30 min (Table 1, Fig. 1).

The intracellular killing was only slightly enhanced by normal chicken serum, but chicken serum exerted some bactericidal capacity by itself, even against the gram-positive staphylococci. This capacity could be abolished by heat inactivation of the serum (30 min, 56 C). In some experiments (Table 1, Fig. 1), where normal serum (presumably replete with complement and normal anti-

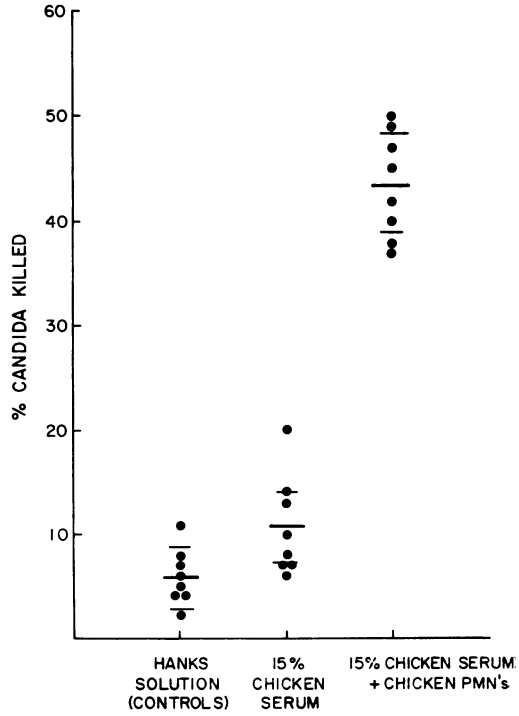

FIG. 3. *Killing of Candida albicans by chicken polymorphonuclear leukocytes (PMN) within 1 hr. The dots represent the results of single experiments; the lines indicate mean and standard deviation.*

body) was used, some bacteria were probably dead when phagocytized. The results, however, suggest that more (probably most) of them died in the PMN. Where heat-inactivated sera were used, the bacteria seemed to have remained viable until phagocytized. In fact, they seemed to have been replicating slightly in the suspending medium (Fig. 1). Killing of the bacteria in the PMN suspensions could not be attributed to the chicken eosinophil PMN, since eosinophils were completely absent (less than 1%) in some experiments (e.g., experiment 141, Fig. 1) without significantly different results.

Chicken PMN also proved to be quite effective in candidacidal assays (Fig. 3). The ratio of killed-to-vital yeasts was uniformly about 40:60, and this ratio differed clearly from the controls of yeasts incubated only with normal chicken serum. These results are quite comparable to those obtained with normal human PMN rich in MPO, but differ from those obtained with PMN from a patient lacking MPO (8). Considering that, due to intracellular digestion, some of the killed *Candida* might be undetected, the decrease of viable *Candida* in the PMN experimental groups could be estimated to be at least 80%. Chicken serum on its own does not decrease the number of viable cells significantly (Fig. 3). Its effect appears to consist mainly in facilitating the aggregation of the *Candida* or their ingestion by the PMN. Again our findings cannot be attributed to the eosinophil PMN. These cells were completely lacking in some experiments, and direct microscopic observations revealed ingested *Candida* in almost every fourth PMN after 30 min of incubation.

DISCUSSION

The mechanisms by which PMN of various species ingest and kill microorganisms have been under renewed investigation for the past several years. These killing mechanisms are inherently complex and probably include various granular contents such as lysozyme (2), cationic proteins (18), and MPO (2). Putatively, the fall in intravacuolar *p*H may also contribute to the microbicidal function of the PMN (3). The proposed antimicrobial system consisting of MPO and H_2O_2, which appears to be activated during phagocytosis as a result of stimulated metabolic activities (13), is one of the most extensively studied killing mechanisms, and it is now known that the presence in vitro of an oxidizable cofactor increases its microbicidal activity (7).

One in vivo method used to study the contribution of MPO has been the addition of toxic substances to phagocytizing whole cells to inhibit peroxidase action. Thus, the addition of azide appears both to inhibit MPO and to lessen the bactericidal capability of PMN (8). However, other workers have found that, by using ascorbic acid, MPO-H_2O_2-halide reactions in the PMN can be inhibited without any loss in bactericidal function (12). A disadvantage of such systems is the difficulty of evaluating the effects of the inhibitory substances on MPO, or possibly also on other factors unrelated to MPO.

Other in vivo methods for studying MPO function have relied on defective PMN from patients with chronic granulomatous disease. The lessened bactericidal abilities of such cells have been documented (5, 6), as well as their improved bactericidal function after introduction of an H_2O_2-generating system into the leukocytes (1). As it happens, patients with chronic granulomatous disease are rare, and the availability of their PMN for research is, therefore, limited.

Still, for MPO various avenues of approach are available, whereas it has heretofore been possible to study other possible killing mechanisms independent of MPO only after they have been extracted from the granules and purified. The advantages of leukocytes naturally lacking MPO and obtainable in sufficient quantities seem obvious. Avian PMN fulfill these qualifications and provide an opportunity to study factors other than MPO that may contribute to antimicrobial function.

The first step in such a study was to examine the microbicidal activity of these leukocytes because, to our knowledge, the killing abilities of chicken PMN had not been documented. Our experiments suggest that avian PMN, although lacking MPO, are quite capable of ingesting and killing microorganisms. Moreover, they display their killing action against microorganisms thought to be killed in human PMN with the aid of MPO. Our results support the hypothesis that substances or mechanisms other than MPO may be powerful factors for antimicrobial effects in PMN.

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