Defective Polymorphonuclear Leukocyte Metabolism and Function in Canine Cyclic Neutropenia

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Humans and grey collie dogs with cyclic neutropenia are known to suffer from an increased rate of bacterial infection. Because of the previously described microanatomic abnormalities of lysosome formation found in the polymophonuclear leukocytes (PMNs) of dogs with canine cyclic neutropenia, studies of these cells were undertaken. PMNs from grey collie dogs were found to have significant metabolic and functional abnormalities when compared with normal collie PMNs. These included abnormally increased postphagocytic C3-glucose oxidation, decreased iodination of trichloroacetic acid-precipitable protein in the resting and phagocytizing state, decreased levels of intracellular myeloperoxidase, and a bactericidal defect against a variety of bacteria. Phagocytosis was normal. These abnormalities appear to differ from those previously described in the PMNs of patients with chronic granulomatous disease of childhood and the Chediak-Higashi syndrome and more closely resemble those seen in hereditary myeloperoxidase deficiency. Thus, the studies reported here demonstrate defective PMN function in a disease state previously believed to be a model only of periodic hematopoiesis.

Cyclic Neutropenia is a hematologic disorder characterized by periodic profound neutropenia which occurs in both human beings and grey collie (GC) dogs. Afflicted humans and dogs are subject to an increased susceptibility to infection. These infections have been attributed to the periodic quantitative decrease of peripheral circulating phagocytes characteristic of this disease. The purpose of the present studies was to determine if there is also a qualitative defect of phagocyte function which might account in part for this increased frequency of infection.

MATERIALS AND METHODS

Animals

All grey collies (GC) studied (four males and three females) were at least 4 mo of age, and none were obviously infected or receiving antibiotics on days of experimentation. Five adult purebred normal collies (NC), three males and two females, 6–18 mo of age, were used as controls.

Phagocyte Preparation

Daily white blood cell (WBC) and differential counts were obtained in all GCs, confirming cyclic neutropenia. Because of the cyclic fluctuation of the blood leukocyte count in this disease, the 12-day cycle was divided in two parts. Days 1–6 of the cycle were considered “ascending” phase and days 7–12 “descending” phase, where day 1 was defined as the first day in which total PMN count was < 500/cu mm. The average peripheral WBC and differential counts of GC dogs in
Table 1. Peripheral WBC and Differential Counts of Grey Collie Dogs on Days of Experimentation

<table>
<thead>
<tr>
<th>Dogs</th>
<th>WBC*/cu mm</th>
<th>PMNs</th>
<th>Bands</th>
<th>Lymphocytes</th>
<th>Monocytes</th>
<th>Eosinophils</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCt ascending (21)§</td>
<td>11480 ± 2470</td>
<td>40.6 ± 6.6</td>
<td>1.3 ± 0.6</td>
<td>37.0 ± 5.0</td>
<td>17.8 ± 3.2</td>
<td>4.0 ± 1.2</td>
</tr>
<tr>
<td>GC descending (25)</td>
<td>9885 ± 1020</td>
<td>53.2 ± 5.4</td>
<td>0.3 ± 0.2</td>
<td>34.2 ± 4.8</td>
<td>8.2 ± 1.3</td>
<td>4.0 ± 0.8</td>
</tr>
<tr>
<td>NC (25)</td>
<td>14300 ± 480</td>
<td>39.5 ± 2.0</td>
<td>0.2 ± 0.1</td>
<td>40.3 ± 1.4</td>
<td>10.2 ± 0.7</td>
<td>10.3 ± 1.9</td>
</tr>
</tbody>
</table>

*Average counts ± SE.
†GC, grey collie; NC, normal collie.
§Ascending phase, days 1–6 of cycle; descending phase, days 7–12.
§Number in parentheses refers to number of observations.

either ascending or descending cycle phases were similar to each other and to NC averages on the days of experimentation (Table 1). At various times in the dogs’ cycles, leukocytes were separated from heparinized peripheral blood by previously reported methods. Contaminating red blood cells were lysed by 20 sec exposure to 0.2% saline. After several washes, the cells were resuspended in Hanks’ balanced salt solution (HBSS) at a concentration of 10 x 10⁶ PMNs per ml. Contaminating monocytes and eosinophils never exceeded 15% of the leukocyte population, while PMNs made up at least 70% of the cells. Trypan blue exclusion always revealed greater than 95% viability at this point.

Phagocytic and Bactericidal Assays

Bactericidal assays were performed by the method of Hirsch and Strauss with previously described modifications. Incubation mixtures contained 5 x 10⁶ PMNs and 10% NC serum previously collected and stored at –70°C. (Since preliminary studies demonstrated that GC serum supported normal phagocytosis by NC and GC PMNs, NC serum was used throughout these experiments because of its greater availability.) Washed overnight cultures of Staphylococcus aureus, Streptococcus fecalis, and Escherichia coli were used in these assays at a ratio of approximately four bacteria per PMN. Aliquots of incubation mixture were removed at designated intervals with a calibrated wire loop, diluted with sterile water to lyse PMNs, and transferred to pour plates which were counted for bacterial colonies 24-48 hr later. Results were expressed as per cent of inoculated bacteria surviving at each period, calculated by dividing the number of viable colonies present at each time by the number of colonies present in the cell-free control at time zero.

The phagocytic uptake of ¹⁴C-radiolabeled heat-killed S. aureus was measured by a previously reported method in which the ratio of bacteria to PMN was 5:10:1. Percentage phagocytic uptake was calculated as: (average cell associated cpm/total cpm added) x 100. Previously, it had been shown that the radioactivity associated with these carefully washed cells closely agreed with other measures of phagocytosis.

Metabolic Studies

C₁-glucose oxidation in GC and NC PMNs was assayed as described previously with some modifications. Twenty-five milliliter Erlenmeyer flasks containing 5 x 10⁶ PMNs in 1 ml of HBSS and 500 nCi of ¹⁴C₁-glucose (New England Nuclear, Boston, Mass.; specific activity, 8.2 mCi/m mole) in 0.5 ml HBSS received either 0.5 ml HBSS (resting samples) or 0.5 ml washed latex particles (average diameter, 1.1 μ; Dow Chemical Co., Midland, Mich.), previously diluted 1: 5 in HBSS (phagocytic samples). The final particle to PMN ratio was approximately 400: 1. The flasks were immediately capped with rubber stoppers (Kontes, Vineland, N. J.), and plastic centerwells (Kontes) were filled with 0.2 ml fresh 10% KOH via syringe. The flasks were placed in a 37°C shaking waterbath for 60 min at which time 1 N HCl was injected through the stopper into the reaction mixture. The centerwell was removed, placed in 10 ml of Aquasol (New England Nuclear), brought to neutrality by the addition of 0.4 ml 3% acetic acid, and radioactivity assayed in a scintillation counter (Beckman LS 250, Fullerton, Calif.)

Iodination by resting and phagocytizing PMNs was measured by the technique of Pincus and Klebanoff. PMNs were incubated alone or in the presence of heat-killed S. aureus at a ratio.
of 100 organisms: PMN. Reaction mixtures contained $5 \times 10^6$ PMNs, 10% NC serum, 200 nCi of carrier-free $^{125}$I (New England Nuclear), and 2 nmoles of cold NaI, and were rotated at 10 rpm (Fisher Rotorack, Pittsburgh, Pa.). After 1 hr incubation, cold trichloroacetic acid (TCA) was added to the tubes which were centrifuged at 2000 g for 10 min. The resulting precipitate was washed three times with 10% TCA and protein-associated radioactivity measured in a Packard Tri-carb Scintillation Counter (Model 2001, Downer's Grove, Ill.)

**Enzymatic Measurements in Cellular Lysates**

Cell suspensions ($10 \times 10^6$ PMNs per ml) in HBSS were freeze thawed five times, yielding a crude lysate which was stored at $-70\degree C$ pending enzymatic determinations.

To assess myeloperoxidase (MPO) activity, Kimball's' modification of Baggioni's' technique was employed. One-tenth milliliter of lysate was added to 2.5 ml of a mixture consisting of 1 part 0.1% o-tolidine (Fisher, Fairlawn, N.J.) in ethyl alcohol, 1 part 10% Triton X100 (Rohr and Hass, Phila., Pa.) in water, 1 part 1.5 mM H$_2$O$_2$ in water, and 10 parts 0.1 M sodium citrate buffer, pH 5.0. After incubation at room temperature for 6 min, 0.1 ml of 10 N NaOH was added. Absorbance at 435 nm was measured in a spectrophotometer (Gilford Model 240, Oberlin, Ohio) and results expressed as microgram equivalents of a standard horseradish peroxidase (Worthington Biochem. Corp., Freehold, N.J.) per milligram lysate protein.

Lysozyme activity in the lysates was quantitated by Litwack's' method using a suspension of Micrococcus lysodeicicus (Worthington) in 0.06 M sodium phosphate buffer, pH 6.2. The decrease in absorbance at 645 nm over a 3-min period produced by 0.3 ml of the lysate was expressed as microgram equivalents of a standard egg-white lysozyme (Worthington) per milligram lysate protein.

Protein concentration of the lysates was estimated by the micro-Folin technique of Lowry.' Purified human IgG (Mann Research Lab., New York, N.Y.) was used as the standard.

![Fig. 1. Staphylocidal capacity of normal collie (NC) and grey collie (GC) PMNs from both ascending (GC ↑) and descending (GC ↓) phases of the dogs' cycles. Significantly fewer (±SE) S. aureus survived after incubation with NC PMNs than after incubation with GC PMNs in either cycle phase (p < 0.005 at each time). Survival of S. aureus did not vary with GC cycle phase except after 1 hr incubation when significantly more (p < 0.005) bacteria survived in the ascending than descending GC cycle phase. Each assay was performed with $5 \times 10^6$ PMNs and a bacteria to leukocyte ratio of 4:1. Statistical analysis of Student's t test. The number in parentheses refers to the number of triplicate experiments performed at each time.](image-url)
RESULTS

Bactericidal Assays

The staphylocidal activity of GC and NC PMNs was measured at $\frac{1}{2}$, 1, 2, and 4 hr after the incubation of these cells with bacteria. In addition, the effect of GC cycle phase upon the staphylocidal activity of PMNs from that phase was studied. GC PMNs from both phases of the GC cycle killed significantly fewer staphylococci ($p < 0.005$ at all times) than did the PMNs of NCs (Fig. 1). There was no significant difference in staphylococcal killing between GC cells taken during the ascending and descending phases of the GC cycle except at 1 hr incubation, when GC PMNs from the second or descending half of the cycle were significantly more staphylocidal than those from the first half (30.6 ± 3.6% versus 50.6 ± 5.8% staphylococcal survival, $p < 0.005$).

Experiments using E. coli or S. fecalis as the test organism (Fig. 2) revealed that GC PMNs killed significantly fewer of both organisms at a given period of incubation than did NC PMNs ($p < 0.05$ for each organism at 2 hr incubation and for E. coli at 1 hr).

Phagocytosis

Phagocytic uptake of $^{14}$C-labeled heat-killed staphylococci after 5, 10, and 20 min incubation by PMNs from NC and GC in both phases of their cycles was compared. No significant difference in phagocytic uptake between cells from NC and GC was found, and phagocytosis by GC cells was unaffected by cycle phase (Table 2).

$^{14}$C-glucose Oxidation

Resting and postphagocytic $^{14}$C-glucose oxidation was measured in both GC and NC PMNs (Table 3). There was no significant difference in glucose oxidation between resting NC and GC PMNs. Although the difference in total glucose oxidation after phagocytosis did not reach statistical significance when GC and NC PMNs were compared, a significantly greater increase ($p < 0.05$) in glucose oxidation above resting values ($\Delta$ glucose oxidation) occurred in the GC PMNs after phagocytosis of latex particles than occurred in NC PMNs.

![Fig. 2. Bactericidal capacity of NC (solid lines) and GC (dotted lines) PMNs against S. fecalis and E. coli. Significantly fewer ($p < 0.05$) organisms survived 2 hr incubation with NC than with GC PMNs. After 1 hr incubation, fewer E. coli ($p < 0.05$) survived incubation with NC than GC PMNs. The ratio of organisms to PMNs was 4 : 1. Statistical analysis by Student's t test. Each point represents the average of four triplicate experiments.](image-url)
Submitted by

R. L. B. W. AND J. J. J. M.

Table 2. Phagocytosis of \(^{14}\)C-labeled Staphylococcus aureus by Grey Collie and Normal Collie PMNs

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<tr>
<th></th>
<th>PMNs</th>
<th>5 Min†</th>
<th>10 Min</th>
<th>20 Min</th>
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<tr>
<td>GC ascending phase</td>
<td>—</td>
<td>38.9 ± 5.8 (5)</td>
<td>43.2 ± 3.3 (6)</td>
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<tr>
<td>GC descending phase</td>
<td>—</td>
<td>35.7 ± 5.1 (9)</td>
<td>38.9 ± 5.1 (9)</td>
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<tr>
<td>GC average, both phases</td>
<td>27.1 ± 6.8† (4)</td>
<td>36.9 ± 3.3 (14)</td>
<td>40.6 ± 3.3 (15)</td>
<td></td>
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<tr>
<td>NC control</td>
<td>19.2 ± 9.2† (3)</td>
<td>39.0 ± 3.0 (12)</td>
<td>43.5 ± 3.8 (14)</td>
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*Incubation mixtures contained 5 × 10^6 PMNs in 1 ml HBSS-10% NC serum and heat-killed \(^{14}\)C-labeled S. aureus at a ratio of 5–10 organisms:PMN. After the incubation period, PMNs and non-phagocytized staphylococci were separated by low-speed centrifugation, and cell-associated radiation was measured.
†Incubation period.
§GC, grey collie; NC, normal collie.
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Table 3. Oxidation of \(^{14}\)C-glucose by Grey and Normal Collie PMNs

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<tr>
<td>Collies</td>
<td>n</td>
<td>Resting (nmoles ± SE)</td>
</tr>
<tr>
<td>Grey</td>
<td>8</td>
<td>13.34 ± 4.38</td>
</tr>
<tr>
<td>Normal</td>
<td>8</td>
<td>15.36 ± 3.31</td>
</tr>
<tr>
<td>p value</td>
<td></td>
<td>NS</td>
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*Reaction mixtures contained 5 × 10^6 PMNs in 2 ml HBSS with 500 nCi \(^{14}\)C-glucose and 10 μmoles cold glucose. Phagocytosis was initiated by the addition of latex particles (400:PMN), and the incubation period was 1 hr. Evolved \(^{14}\)CO₂ was trapped in KOH.
†Each experiment a triplicate average.
‡Student’s t test. NS (not significant) = p > 0.05.

Iodination

Iodination of heat-killed staphylococci by GC and NC PMNs as measured by \(^{125}\)I fixation revealed no significant difference between GC PMNs of opposite cycle phase, but a marked deficiency in GC PMN iodination compared with NC cells (Table 4). Total postphagocytic iodination by GC PMNs was markedly less than that by NC cells (p < 0.001), and iodination by resting PMNs was also significantly less (p < 0.05) in GC than in NC cells.

Intracellular Enzymes

The activity of MPO, lysozyme, and the protein concentration of lysates of suspensions of GC and NC PMNs adjusted to a concentration of 10 × 10^6 PMNs per ml were measured (Table 5). There was significantly less MPO activity per milligram protein present in the lysates of GC cells than was present in the NC lysates (p < 0.001). This deficiency was present equally in both ascending and descending phases of the GC cycle. There was a suggestive decrease in lysozyme activity per milligram protein in the GC lysates compared with NC lysates, but this difference was not statistically significant.

DISCUSSION

The data reported here demonstrate that, although GC PMNs ingest bacteria normally, they show definite functional and metabolic abnormalities. These in-
include decreased ability to kill at least three different species of bacteria, enhanced postphagocytic stimulation of C₁-glucose oxidation, and markedly decreased ability of resting and phagocytizing GC PMNs to iodinate protein. GC PMNs also possess significantly less MPO activity than NC PMNs. These defects appear to be specific to canine cyclic neutropenia, since parallel abnormalities were not found in studies of the PMNs of three patients with human cyclic neutropenia.

These defects appear in PMNs from both the ascending and descending phases of GC cycles. In all aspects studied, there have been no significant differences between PMNs taken during either phase of the cycles except in the bactericidal studies, in which there is a suggestion that cells taken during the first half of the cycle might be less bactericidal, especially at short incubation periods. Although increased numbers of monocytes are seen in GC peripheral blood in the ascending cycle phase (Table 1), and monocytes have been demonstrated to be less efficient in phagocytosis and hence bacterial killing than PMNs, it is unlikely that this relative monocytosis is responsible for the increased bactericidal defect noted, since in no instance did contaminating monocytes constitute more than 15% of the leukocyte mixture. Additionally,
morphologically immature PMNs, which are known to possess decreased phagocytic ability, are occasionally found in GC peripheral blood, especially in the early ascending phase. However, these cells are found in relatively low numbers (Table 1), making their role in the increased bactericidal defect of the ascending phase questionable. Furthermore, no difference in phagocytosis could be demonstrated between PMNs from the ascending or descending phases of the GC cycle (Table 2); this would be expected if a phagocytically less efficient cell (monocyte or eosinophil) or immature cell (band) were the cause of the difference in bactericidal capacity between cycle phases. It should be noted that toxic neutrophils, which are known to possess a variety of functional and metabolic abnormalities, were not observed in the blood of these dogs in either cycle phase during the course of these studies.

The pattern of abnormalities detected in the GC PMNs best fits that which would be expected if there was a defect in the MPO/halide/hydrogen peroxide bactericidal system first elucidated by Klebanoff. In this system, the combined action of hydrogen peroxide, MPO, and a halide within the phagolysosome leads to bacterial death. The hydrogen peroxide produced in large quantities after phagocytosis is associated with increased C₆-glucose oxidation via the hexose monophosphate shunt. It has been suggested that the final event leading to bacterial death is the deamination or decarboxylation of bacterial cell wall protein by activated halide complexes.

Defects in at least two different phases of this system have been documented: one in the phagocytes of patients with chronic granulomatous disease of childhood which produce inadequate amounts of hydrogen peroxide to allow normal halide utilization, and the other in PMNs from patients with hereditary myeloperoxidase deficiency which lack the enzyme necessary for the formation of activated halide complexes. PMNs from both groups of patients show markedly decreased ability to fix iodine after phagocytosis of bacteria. The PMNs from both groups have been demonstrated to have bactericidal and fungicidal defects.

The experiments reported here demonstrate that PMNs from GC dogs with cyclic neutropenia possess a similar defect that leads to markedly decreased ability to iodinate bacterial proteins and to kill bacteria after ingestion. However, unlike CGD PMNs, grey collie PMNs are unable to kill efficiently a non-catalase-producing bacterium, S. fecalis, and also unlike CGD PMNs they show normal, if not supranormal, stimulation of C₆-glucose oxidation after phagocytosis. Thus, the GC defects do not closely parallel those of CGD. Instead, they seem more closely related to those seen in human hereditary MPO deficiency, even though the MPO activity of PMNs from GC dogs is about 50% normal, and the PMNs of MPO-deficient patients have essentially no demonstrable MPO activity.

MPO-deficient PMNs have been demonstrated to have normal phagocytosis, increased C₆-glucose oxidation after phagocytosis, and abnormally depressed iodination of TCA-precipitable protein during phagocytosis, findings identical with those seen in GC PMNs. It has been suggested that the increased C₆-glucose oxidation seen in human MPO-deficient PMNs during and after phagocytosis is a compensatory event, since increased glucose oxidation seems
directly linked with increased \( \text{H}_2\text{O}_2 \) production and \( \text{H}_2\text{O}_2 \) itself is bactericidal.\textsuperscript{35,36} The PMNs from GC dogs show a similar compensation with significantly increased \( \text{C}_1\)-glucose oxidation after phagocytosis in the face of markedly decreased iodinating ability. This may also explain the relatively modest bactericidal defect demonstrated in GC and MPO-deficient PMNs.\textsuperscript{35,36}

It has been demonstrated that GC PMNs are morphologically abnormal at the electron-microscopic level, containing an overabundance of at least one substance, glycogen.\textsuperscript{37} In addition, abnormalities of lysosomal formation have been observed, characterized by peculiar ring-shaped peroxidase-positive structures.\textsuperscript{37} These microanatomic abnormalities potentially may adversely affect formation of the phagolysosome and extrusion of lysosomal contents into the phagocytic vesicle. Such anatomic abnormalities might also explain why iodination in these cells is so markedly reduced, even in the presence of 50\% normal MPO activity. Though the MPO in these cells may be available for enzymatic analysis after freeze-thawing, abnormal granule morphology may make the enzyme inaccessible for iodination reactions within the phagolysosome.

The Chediak-Higashi syndrome (CHS) is a disease in which abnormal lysosomal function and degranulation have been demonstrated. CHS has been described in man\textsuperscript{38} and a variety of animals\textsuperscript{39} and is characterized by pigmented dilution, the presence of giant lysosomal inclusions within a variety of cell types including blood leukocytes, and an increased predisposition to infection.\textsuperscript{38} Detailed studies have documented decreased PMN bactericidal capacity against several bacterial species, including \textit{S. aureus}, \textit{S. fecalis}, and \textit{E. coli}, and decreased concentrations of MPO within CHS PMNs.\textsuperscript{40} Thus, the grey collie syndrome might seem related to CHS in view of the dogs' pigmented dilution, PMN bactericidal deficiency, and structural abnormalities of PMN lysosomes at the electron-microscopic level. However, the grey collie syndrome differs from CHS in that giant lysosomes are not found within blood leukocytes, and the GC PMN bactericidal defect is prolonged and present not simply for the first 20 min after phagocytosis, as has been demonstrated in CHS.\textsuperscript{11} Additionally, in the grey collie syndrome the rate of iodination by PMNs in both resting and phagocytizing states is decreased rather than increased as noted in CHS.\textsuperscript{11} Finally, in CHS, \( \text{C}_1\)-glucose oxidation by PMNs is markedly increased above normal in the resting state,\textsuperscript{11} while it is normal in the grey collie syndrome. Thus, the grey collie syndrome and CHS, while sharing some common facets, are clearly distinct from one another with respect to the PMN functional and metabolic abnormalities which characterize each of them.

While previous workers have demonstrated the applicability of studying the grey collie syndrome of cyclic neutropenia as a model of human cyclic neutropenia, the studies presented here suggest that, in addition to the presence of a periodic quantitative PMN deficiency, a qualitative PMN functional defect is also present. This defect closely resembles that seen in the PMNs of patients with hereditary MPO deficiency. Thus, these animals may also prove to be a useful laboratory model for the study of functional defects in PMNs.

**ACKNOWLEDGMENT**

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REFERENCES

26. Lehrer RI, Cline MJ: Leukocyte myeloperoxidase deficiency and disseminated can-
Defective polymorphonuclear leukocyte metabolism and function in canine cyclic neutropenia

MJ Chusid, JS Bujak and DC Dale