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# Effect of Bone Marrow Suppression on Granulocyte Opsonin Levels (39153)[1]

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(Introduced by G. G. Jackson)

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Transient bone marrow suppression following anti-tumor and immunosuppressive therapy is a common occurrence. Techniques are now available to provide granulocyte transfusions which may prevent the bacterial infections that constitute the most serious complication of marrow failure. The purpose of the present study was to evaluate the effect of marrow suppression on serum granulocyte opsonin levels in dogs. This should provide useful information about the environment in which transfused granulocytes will be expected to function.

*Materials and methods. Induction of neutropenia.* Mongrel dogs weighing between 10 to 25 kg were dewormed and immunized against hepatitis and distemper. Following a 1-week period of observation, they were given cyclophosphamide (CY) (Mead-Johnson Laboratories, Evansville, Ind.) intravenously at a dose of 40 mg/kg. Parenteral Ringers solution was used to maintain hydration during periods of anorexia. Absolute granulocyte levels were determined from the white blood cell (WBC) and differential counts. As shown previously (1), the granulocyte levels reached their nadir 4 days after the administration of CY. The studies were terminated 4 or 5 days after CY treatment when fever and bacteremia developed in these infection susceptible animals.

*Granulocyte opsonin assay.* WBC preparations were obtained from heparinized canine blood by sedimenting the red blood cells (RBC's) with Plasmagel (Roger Bellon Laboratories, Neuilly, France) for 30 min at 37°. The buffy coat was removed from the supernatant fluid by centrifugation at 400*g* for 10 min. The WBC's were washed and suspended in Hanks balanced salt solution (HBSS) supplemented with glucose at a concentration of 2 mg/ml. The WBC suspension was adjusted to contain $5 \times 10^6$ granulocytes/ml and 40% serum from either normal or CY treated dogs. Serum heated to 56° for 30 min prior to assay was employed to demonstrate heat liability.

The WBC suspension (0.4 ml) was combined with 0.4 ml of washed Bakers' yeast suspension containing $4 \times 10^7$ yeast particles/ml and incubated in a shaker-water bath for 30 min at 38°. Following incubation, each tube was supplemented with 0.1 ml of a 0.9% EDTA–saline solution followed by centrifugation for 5 min at 800*g*. The pellet was transferred to a clean microscope slide, air-dried, fixed in methanol for 5 min, and stained with Wright's-Giemsa. In the evaluation of phagocytosis, a minimum of 200 granulocytes were examined for yeast uptake.

Two methods of evaluating phagocytosis were initially compared: (i) determining the percentage of granulocytes with one or more yeast particles, and (ii) calculating the average number of yeast particles per granulocyte. The former method proved to be the most consistent and was used throughout this study. This value is referred to as the phagocytic index and is used as a measure of the opsonic or phagocytosis stimulatory capacity of the test serum.

*Immunoglobulin and complement assays.* IgG and C3 ($\beta_1$C) levels were determined by the agar immunodiffusion method of Mancini (2) using rabbit antisera specific for dog IgG and C3. Hemolytic complement levels were determined by standard methods (3) and expressed as CH50 units.

*Results. Granulocyte opsonin.* In the presence of fresh normal dog serum, the

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mean phagocytic index relative to normal dog granulocyte uptake of yeast particles was 62 ($n$ = 65, range = 58–66, S.D. ± 2.65). Heated normal serum (56°, 30 min) had a significantly lower ($P < 0.01$) phagocytic index (mean = 52, $n$ = 9, S.D. ± 5.48). By contrast, in the absence of serum the value was 26 ($n$ = 6, S.D. ± 3.99).

Figure 1 shows the level of opsonic activity of serum from 6 of the CY treated dogs as assayed with normal dog granulocytes. By 24 hr following CY administration the opsonin level was significantly elevated ($P < 0.005$). By the second day, the opsonin level appears to have reached a plateau that continues through the period of observation (4–5 days post-CY). The elevation in serum granulocyte opsonic activity remained statistically significant ($P < 0.005$) throughout this period.

The peripheral neutrophilic granulocyte level in all 6 CY treated dogs was below 600/mm$^3$ on the fourth post-treatment day, but on the first post-treatment day at a time when the serum opsonin level was already elevated, the granulocyte level had not changed significantly. The average granulocyte count on Day 0 was 8413 cells/mm$^3$ and on Day 1 was 8428 cells/mm$^3$.

The opsonic activity of heated (56°, 30 min) and unheated serum from normal and CY treated dogs were compared (Table I) in a system using normal dog granulocytes. An increase in the phagocytic index of fresh serum from CY treated dogs is shown. Heat inactivation of both normal and CY serum resulted in reduction of the phagocytic index to similar levels, demonstrating that the increase in opsonin of CY serum is due to an elevation in the heat labile component. Serum from two cyclophosphamide-treated dogs were tested to determine the stability of serum granulocyte opsonins to heating at 50° for 30 min (a measure to differentiate the alternate from the classical complement pathway). Both sera had decreases in opsonin activity equal to that which occurred with heating to 56°.

In order to block the classical complement pathway selectively, EGTA (10 m$M$) and $MgCl_2$ (7 m$M$) were added to the serum prior to the phagocytosis assay (4). Calcium chelation failed to depress the phagocytic index of normal or post-CY serum, suggesting that the alternate complement pathway was responsible for the heat-labile opsonin.

Bone marrow suppression as demonstrated by profound neutropenia was also produced in one dog by 1350 rad of whole body irradiation and in another dog by busulfan therapy (total oral dose, 848 mg). Both dogs developed significant elevations of serum granulocyte opsonins. The phagocytic index was 75 or 76 for both dogs throughout the period of neutropenia.

In order to evaluate the influence of stress on the serum granulocyte opsonin levels four dogs were examined 1–3 days following surgical procedures which resulted in varying degrees of blood loss. In all instances phagocytic indices remained within normal limits (range 62–64).

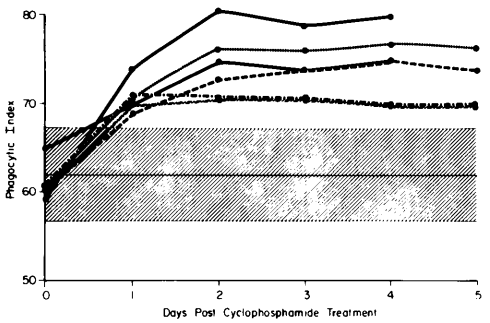


FIG. 1. Level of granulocyte opsonin in the serum of cyclophosphamide treated dogs. The shaded area represents ±2 S.D. around the mean of the phagocytic indices of normal dog serum.

TABLE I. GRANULOCYTE OPSONIN LEVEL BEFORE AND AFTER CYCLOPHOSPHAMIDE TREATMENT.[a]

| Days post-CY | Dog | Phagocytic index | | | |
|---|---|---|---|---|---|
| | | NS | CYS | ΔNS | ΔCYS |
| 0 | 3 | 63 | — | 56 | — |
| 1 | 3 | 60 | 70 | 53 | 53 |
| 2 | 3 | 65 | 75 | 57 | 51 |
| 3 | 3 | 60 | 74 | 57 | 55 |
| | 7 | 60 | 77 | 50 | 42 |
| 4 | 3 | 60 | 75 | 57 | 57 |
| | 7 | 65 | 75 | 51 | 50 |

[a] The opsonin level measured by the phagocytic index was determined for normal dog serum (NS), heated normal dog serum (ΔNS), serum from cyclophosphamide treated dogs (CYS), and heated serum from cyclophosphamide treated dogs (ΔCYS). Normal dog granulocytes were used in all of these determinations.

*Phagocytic ability of granulocytes.* The response of granulocytes obtained from CY treated dogs to heated and unheated, normal, and CY serum are shown in Fig. 2. It is apparent that in the 1–4 day interval following CY treatment there was a progressive deterioration in the phagocytic ability of the granulocytes obtained from CY treated dogs regardless of the serum used. However, heated serum when used with CY cells from dog 3 produced a lower phagocytic index than unheated serum. There did not appear to be a significant difference between the response elicited by fresh normal and CY serum inspite of the differences observed when normal granulocytes were used.

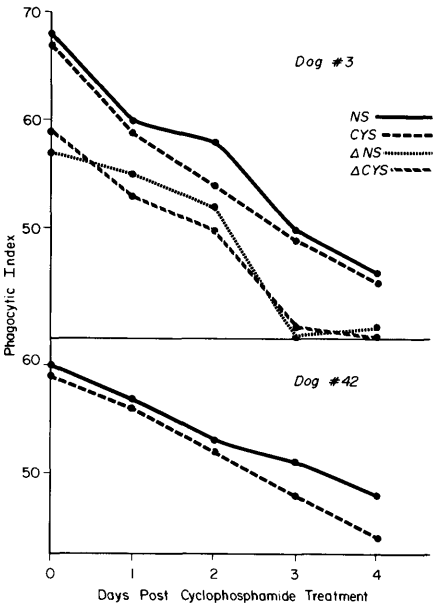


FIG. 2. Phagocytic ability of granulocytes from cyclophosphamide treated dogs using the following kinds of serum to supply opsonic activity: Normal serum (NS), heated normal serum (ΔNS), serum from the cyclophosphamide treated dog furnishing the granulocytes (CYS), and heated cyclophosphamide serum (ΔCYS).

*Immunoglobulin and complement.* IgG, C3, and hemolytic complement assays were performed in three dogs prior to, and for 5 days following, CY therapy (Table II). Hemolytic complement appeared to increase modestly, but the increase did not correlate with the prompt rise in granulocyte opsonin levels. IgG levels were essentially unchanged in two dogs (3424 and 3456) while a decrease occurred in dog 3455 over the 5 day post-CY period. C3 values followed no consistent pattern and failed to correlate with the opsonin levels.

*Discussion.* CY is known to act as an alkylating agent primarily toxic to rapidly proliferating cells (4). Significant serum levels of CY are not observed 24 hr after administration (5, 6), indicating that the drug is cleared within that period. Its effect on

TABLE II. SERUM LEVELS OF COMPLEMENT, IgG, C3 ($\beta_1$C), AND GRANULOCYTE OPSONIN IN THREE DOGS PRIOR TO AND FOLLOWING CYCLOPHOSPHAMIDE THERAPY.[a]

| Serum levels | Days post-cyclophosphamide therapy | | | | | | |
|---|---|---|---|---|---|---|---|
| | −1 | 0 | 1 | 2 | 3 | 4 | 5 |
| Dog 3424 | | | | | | | |
| Complement | 24 | 24 | 22 | 26 | 22 | 24 | 32 |
| IgG | 40 | 39 | 40 | 39 | 39 | 34 | 23 |
| C3 ($\beta_1$C) | 7.8 | 7.3 | 7.3 | 7.3 | 7.0 | 7.8 | 8.6 |
| Granulocyte opsonin | 60 | 61 | 71 | 74 | 74 | 75 | 74 |
| Dog 3455 | | | | | | | |
| Complement | 28 | 26 | 24 | 26 | 29 | 31 | 26 |
| IgG | 26 | 29 | 29 | 21 | 21 | 16 | 16 |
| C3 ($\beta_1$C) | 12.0 | 12.0 | 12.0 | 12.0 | 13.0 | 13.0 | 13.9 |
| Granulocyte opsonin | 61 | 61 | 72 | 71 | 71 | 70 | 70 |
| Dog 3456 | | | | | | | |
| Complement | 28 | 26 | 27 | 33 | 37 | 39 | 40 |
| IgG | 29 | 29 | 25 | 29 | 29 | 29 | 29 |
| C3($\beta_1$C) | 12.0 | 11.5 | 9.6 | 11.5 | 12.0 | 12.0 | 12.0 |
| Granulocyte opsonin | 63 | 60 | 69 | 70 | 70 | 69 | 69 |

[a] The values shown represent the following measurements: complement = CH50 units; IgG = mg/ml; C3 ($\beta_1$C) = mg/ml; and granulocyte opsonin = phagocytic index (see text).

bone marrow in the dose used in this study is to transiently suppress hematopoiesis. Nondividing cells such as mature granulocytes are not significantly affected. This appears to be borne out in the present study where granulocytes have near normal phagocytic ability shortly after CY treatment, but demonstrate progressively deteriorating phagocytic ability over a 4 day period. The deterioration is probably due to the increasing average age of the cells during a period when the marrow is not producing new granulocytes. It would be anticipated that the decreasing cell function increases the risks of pyogenic infection beyond what would be expected when considering only the numbers of circulating granulocytes.

The serum opsonin levels for yeast rose in all six animals following CY administration, and in the single dogs treated with X-ray and busulfan. The increase was largely, if not completely, due to heat-labile opsonin. Although other studies (8) have shown that heat-labile opsonins are components of the complement system, there was no significant increase in hemolytic complement or C3 levels in the CY treated dogs. The finding that the heat-labile fraction of the serum opsonins was inactivated by heating to only 50° for 30 min and the failure of calcium chelation with EGTA (4) to depress the serum opsonic activity, suggests that the alternate complement pathway is responsible for the observed change. At the present time, assays for the alternate pathway components in dogs are not available. Other investigations, however, have shown that in humans, the alternate complement pathway is involved in the production of heat-labile granulocyte opsonins (9, 10). Miller and Nilsson (11) have shown the importance of C5 as an opsonin for yeast in human serum. Since the role of C5 as a yeast opsonin in canine serum is unknown, it warrants further investigation when techniques are available for isolation and assay.

The increase in opsonin level seen in CY treated dogs appears to be related to bone marrow suppression rather than any specific effect of CY since X-ray and busulfan suppression of the bone marrow had similar effects while the stress of surgery, anesthesia, and blood loss did not increase the levels.

Decrease in the peripheral granulocyte level was not responsible for the increase in serum opsonins since they rose prior to the development of granulocytopenia. However, the opsonin rise could be related to the size of the total granulocyte pool or changes in granulocyte function. Although the mechanism for the increase in serum heat-labile opsonin in marrow-suppressed dogs is unknown, if similar changes occur in marrow suppressed humans, the *in vitro* function of transfused granulocytes should be enhanced.

*Summary.* Levels of serum opsonin for neutrophilic granulocytes were measured in dogs made neutropenic by cyclophosphamide administration. Heat-labile opsonin became elevated within 24 hr following cyclophosphamide ($P < 0.005$) and remained elevated over the 4–5 day period of observation ($P < 0.005$). In contrast, heat stable opsonin was not significantly effected. Bone marrow suppression by X-ray and busulfan also caused serum opsonin levels to increase. Changes in the levels of IgG, C3, and total hemolytic complement during the course of bone marrow suppression did not correlate with the granulocyte opsonin levels. These findings suggest that serum granulocyte opsonin levels respond to bone marrow suppression and may provide an improved environment for the function of transfused granulocytes.

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