Sequential Morphological and Quantitative Changes in Blood and Bone Marrow Neutrophils in Dogs with Acute Inflammation

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ABSTRACT

Blood and bone marrow morphology were studied sequentially in dogs during experimental inflammation induced by intramuscular injection of turpentine.

Depletion of the bone marrow storage pool of mature neutrophils and an increase in mitotic activity and number of early granulocyte precursors were evident within 24 hours. During the next three days, intense granulocytic hyperplasia resulted in replenishment of the bone marrow storage pool.

Neutrophils with foamy vacuolation and increased basophilia of the cytoplasm (toxic neutrophils) were present in the blood by eight hours postinjection. The number of toxic neutrophils paralleled the intensity of clinical signs and changes in rectal temperature but not the number of band neutrophils. This indicates that changes in number of toxic neutrophils in sequential leukograms can be a prognostic indicator in dogs with severe inflammation.

Key words: Blood, bone marrow, neutrophil morphology, toxic neutrophils.

RÉSUMÉ

Cette expérience visait à étudier, de façon séquentielle, la morphologie du sang et de la moelle osseuse, chez des chiens, à la faveur d'une inflammation expérimentale consécutive à l'injection intramusculaire de térébenthine.

Une déplétion de la réserve médullaire en neutrophiles matures, ainsi qu'une augmentation de l'activité mitotique et du nombre des précurseurs des granulocytes initiaux se manifestèrent en moins de 24 heures. Durant les trois jours suivants, une hyperplasie marquée des granulocytes résulta en une normalisation de la réserve médullaire.

Des neutrophiles toxiques qui se caractérisaient par une vacuolisation spumeuse et une basophilie cytoplasmique accentuée, apparurent dans le sang, huit heures après l'injection de térébenthine. Le nombre de neutrophiles toxiques s'avéra proportionnel à la gravité des signes cliniques et aux variations de la température rectale, mais non au nombre de neutrophiles segmentés. Ces constatations indiquent que les changements dans le nombre de neutrophiles toxiques, lors de leucogrammes séquentiels, peuvent fournir un indice pronostique, chez les chiens qui souffrent d'une grave inflammation.

Mots clés: sang, moelle osseuse, morphologie des neutrophiles, neutrophiles toxiques.

INTRODUCTION

Acute inflammation causes changes in the morphology and number of blood neutrophils, as well as neutrophil production and release from bone marrow. Toxic is a term used to describe alterations in neutrophil morphology associated with severe bacterial infections or other inflammatory disorders. Cytoplasmic features of toxic neutrophils in the dog include basophilia, vacuolation and an occasional Döhle body. Giant neutrophils and bizarre nuclear morphology are also seen. These changes have been attributed to depression of granulopoiesis and defective neutrophil maturation during severe toxemic states (1,2). The kinetics of the neutrophil response to acute inflammation in the dog have been studied (3,4). However, little is known about the sequential morphological changes in blood and bone marrow and much of what is known has been derived from clinical cases (1,2). The purpose of this study was to determine the sequential morphological and quantitive changes in blood and bone marrow neutrophils during acute inflammation in dogs.

MATERIALS AND METHODS

Seven mixed-breed dogs (three male, four female) were injected in the left gluteal muscles with 1.0 mL turpentine (Medi-Kay Pharmacal Co., Brookfield, Missouri). Seven control dogs (three male, four female) were injected similarly with 1.0 mL sterile 0.9% saline.

Clinical signs and rectal temperature were recorded daily. Venous blood samples for hematological determinations were collected in potassium EDTA (Vacutainer, Becton-Dickinson, Rutherford, New Jersey) on three days immediately prior to injection. Postinjection blood samples were collected at 8, 16 and 24 hours, and then daily through day 9. Packed cell volume (PCV) was measured by the microhematocrit technique and plasma protein concentration was determined by refractometry. Leukocyte counts were determined by an electronic cell counter (Coulter Model

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Zf, Coulter Electronics Inc., Hialeah, Florida). Blood films were prepared (Uni-Smear Spinner, Coleman Instruments Division, Perkin-Elmer, Oak Brook, Illinois) and stained with modified Wright's stain (Hema-tek, Ames Division, Miles Laboratories, Inc., Elkhart, Indiana). Differential leukocyte counts on 100 cells were done and the presence or absence of toxic change was determined by examining 50 neutrophils on each blood smear. Neutrophils were considered toxic if the cytoplasm was foamy, vacuolated, basophilic or if Döhle bodies were present.

Bone marrow samples were aspirated without anticoagulant from the ilium or humerus of four dogs from each group using an 18 gauge Rosenthal needle. Samples were collected immediately prior to injection and on days 1, 2, 3, 4 and 9 postinjection. Marrow smears were stained with modified Wright's stain and five hundred cells were identified to assess granulocyte morphology and to determine myeloid:erythroid ratio (M:E) and the percentage of granulocytes in the mitotic and maturation pools. Myeloblasts, progranulocytes and myelocytes were included in the mitotic pool; metamyelocytes, bands and segmented neutrophils were included in the maturation pool.

The hematology and bone marrow data were evaluated statistically by 2 treatment * 14 times and 2 treatment * 6 times factorial analyses of variance, respectively, with repeated measures on the last factor. Single degree of freedom contrasts were done to address specific hypotheses (5).

RESULTS

Injection sites of dogs given turpentine were swollen, reddened and warm, but not severely painful by day 1. The size of the lesion increased over the first three to five days into a firm mass involving the entire left gluteal region. Lameness of the turpentine-injected limb, anorexia and depression were noted by eight hours postinjection, were most severe on days 2-3 and had subsided by days 7-8. Average rectal temperatures in turpentine-injected animals were significantly increased at day 1, peaked on day 2 and declined to control values by day 7 (Fig. 1). Clinical signs and increased rectal temperature were not observed in control dogs.

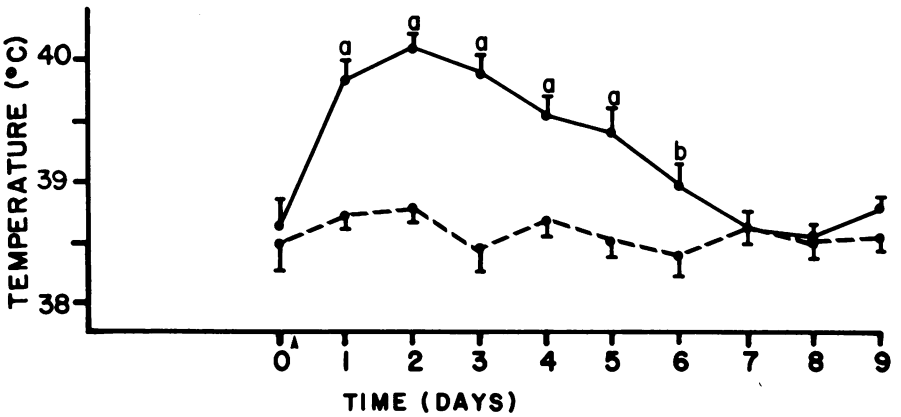

Fig. 1. Body temperature in control (---) and turpentine-injected dogs (___). Each point represents $\bar{x}$ ± SEM, n = 7. Arrow signifies time of injection.
a = means are significantly different ($P < 0.01$)
b = means are significantly different ($P < 0.05$)

Changes in neutrophil number paralleled the total leukocyte count in turpentine-injected dogs. A statistically significant leukocytosis and absolute neutrophilia were observed at eight hours postinjection and persisted through day 6 (Fig. 2). The peak neutrophilia range from 20.5 x 10^9/L to 48.9 x 10^9/L. Immature forms of neutrophils were seen rarely in blood smears of control dogs and in preinjection blood samples. Band neutrophils were increased significantly between 16 hours and day 4 after turpentine injection (Fig. 2).

Basophilia and vacuolation of the cytoplasm were frequent in both band and segmented neutrophils of turpentine-injected dogs (Fig. 3). The vacuoles varied in size and had irregular, indistinct margins giving the cytoplasm a foamy or moth-eaten appearance. The severity of toxic change did not appear to be related to nuclear maturity in that segmented and band neutrophils were both affected. Blue-gray, amorphous, cytoplasmic inclusions or Döhle bodies were seen infrequently. Toxic neutrophils were observed at eight hours and were increased significantly by 16 hours (Fig. 2). The number of toxic neutrophils in turpentine-injected dogs peaked on day 2 and declined to control levels by day 7.

Circulating lymphocytes were decreased significantly by eight hours and remained below control levels through day 9. Eosinopenia began at 16 hours and persisted through day 5. Monocytosis began on day 2, peaked on day 3 and continued through day 6 (Fig. 4).

A mild but statistically significant decrease in PCV occurrred in the turpentine-injected dogs ($P < 0.01$). Average PCV values declined from a preinjection level of 0.43 to 0.39 at day 9. The reduction was not accompanied by significant changes in plasma protein concentration. Packed cell volume and plasma protein concentration for control dogs did not vary significantly during this experiment.

Myeloid:erythroid ratios (M:E) and the percentage of mitotic granulocytes are expressed graphicallly in Fig. 5. The M:E of preinjection bone marrow samples from control and test animals were not significantly different ($\bar{x}$ = 1.4:1 and 1.2:1 respectively). Control and preinjection bone marrow granulocytes averaged 88.4% postmitotic neutrophils and 11.6% mitotic granulocytes. Granulocyte mitoses accounted for 0.2% of the total nucleated cells.

At 24 hours postinjection (day 1), the M:E had not changed in test dogs. However, the percentage of cells in the postmitotic compartment was significantly decreased whereas the percentage of cells in the mitotic pool had increased ($\bar{x}$ = 27.6%). Mitotic figures in granulocyte precursors were increased ($P < 0.05$), comprising 0.6% of the nucleated cells (Fig. 6). Irregular nuclear lobulation or blebbing was observed in several myeloblasts and progranulocytes (Fig. 7). A few neutrophils had doughnut-shaped nuclei. Distinct, clear, circular, intracytoplasmic vacuoles were present in myeloblasts and progranulocytes (Fig. 8).

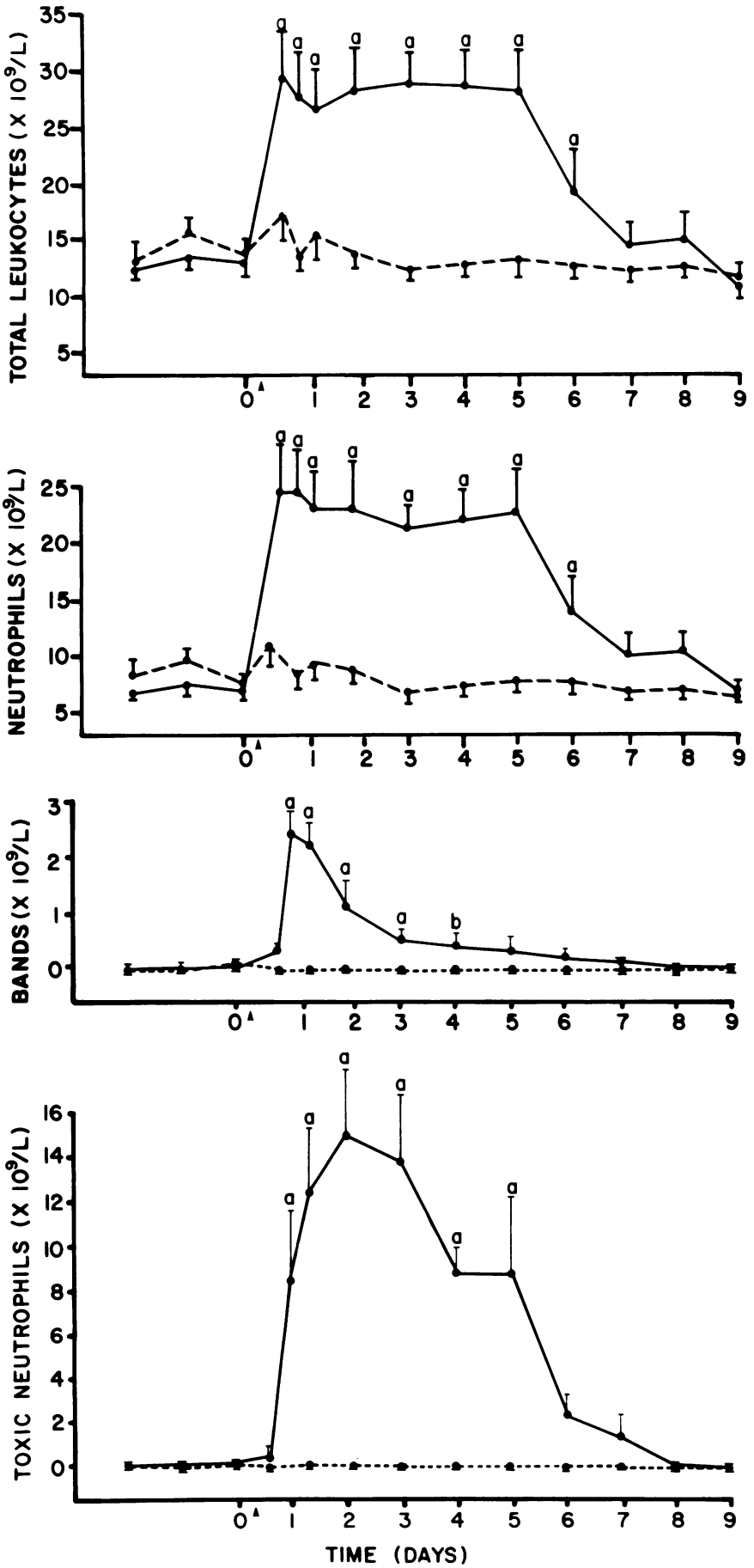

Fig. 2. Leukocyte, neutrophil, toxic neutrophil and band neutrophil counts in control (---) and turpentine injected dogs (___). Each point represents $\bar{x} \pm$ SEM, n = 7. Arrows signify time of injection.
a = means are significantly different ($P < 0.01$)
b = means are significantly different ($P < 0.05$)

Increased cytoplasmic basophilia and foaminess were noted in myelocytes and metamyelocytes, but were more prominent in band and segmented neutrophils.

By day 2, the M:E averaged 21.1:1. The percentage of neutrophils in the mitotic pool had increased to 34.0%. Granulocyte mitotic figures were more numerous (1.5% of nucleated cells, $P < 0.01$). Morphological changes in neutrophil precursors were similar to day 1, except that round, clear, distinct cytoplasmic vacuoles were observed more frequently in myelocytes and metamyelocytes (Fig. 9).

On day 3, M:E remained high ($\bar{x}$ = 20.0:1), 1.1% of the nucleated cells were granulocytes in mitosis ($P < 0.05$) and the percentage of cells in the mitotic pool had declined ($\bar{x}$ = 21.2%). Cytoplasmic vacuoles were less prominent and were seen primarily in myelocytes and metamyelocytes. The numbers of toxic neutrophils and early precursors with lobulated nuclei were decreased.

On day 4, the M:E remained elevated, but the percentage of cells in the mitotic pool ($\bar{x}$ = 14.9%) and number of granulocyte mitotic figures were similar to control values. Morphological abnormalities of the granulocytic series were minimal.

Myeloid:erythroid ratios were approaching control values by day 9. The percentage of neutrophils in the mitotic pool and neutrophil morphology were similar to the control animals.

Significant changes in M:E, percentage of cells in the mitotic pool and neutrophil morphology were not observed in control dogs.

DISCUSSION

In this experiment, intramuscular injection of turpentine caused acute localized inflammation with associated clinical signs, pyrexia and neutrophilic leukocytosis with a left shift and toxic change. Prominent foamy

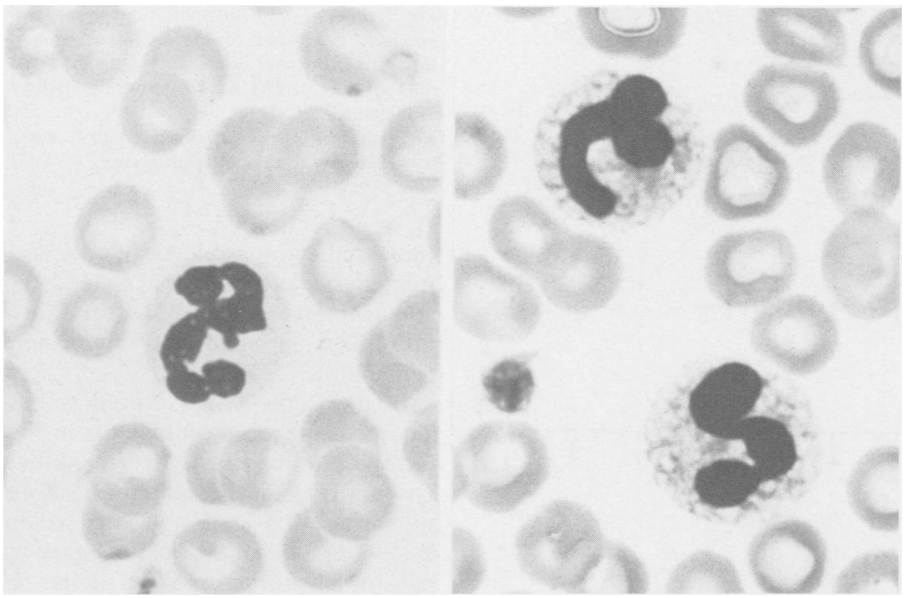

Fig. 3. A) Neutrophil in blood from a control dog. Modified Wright's stain. X1500. B) Toxic neutrophils in blood from a turpentine-injected dog. The cytoplasm is foamy and vacuolated. Modified Wright's stain. X1500.

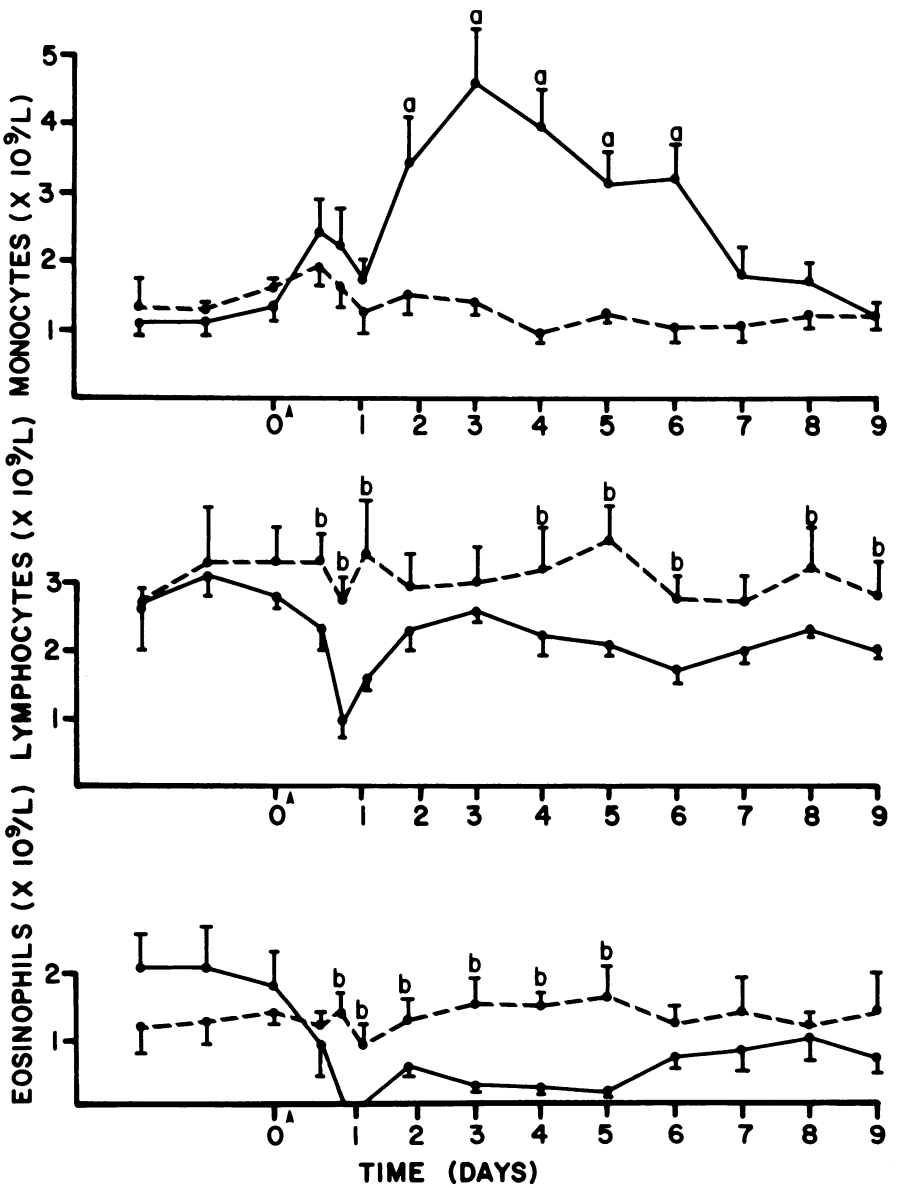

Fig. 4. Lymphocyte, eosinophil and monocyte counts in control (---) and turpentine-injected dogs (___). Each point represents $\bar{x}$ ±SEM, n = 7. Arrows signify time of injection.
a = means are significantly different ($P < 0.01$)
b = means are significantly different ($P < 0.05$)

vacuolation and increased basophilia of neutrophil cytoplasm (toxic changes) were similar to changes described clinically in dogs with severe inflammation (1,2). Döhle bodies were seen infrequently.

Toxic neutrophils were first observed at eight hours, peaked at day 2 and declined gradually to control levels by day 7. The maximum number of toxic neutrophils was variable, but changes in number paralleled the intensity of clinical signs and changes in rectal temperature. Toxic neutrophils began to decline earlier than neutrophil count and total leukocyte count. Band neutrophils peaked earlier and declined more rapidly than did the number of toxic neutrophils. A similar lack of correlation between toxic change and state of nuclear development has been reported in man (6). These findings suggest that changes in numbers of toxic neutrophils rather than band neutrophils are more indicative of the clinical course of inflammation.

Monocytosis, eosinopenia and lymphopenia were also noted in leukograms of turpentine-treated dogs. Inflammation at the injection site may have caused the monocytosis. Due to minimal storage of monocytes in the marrow, the monocytosis of inflammation results from enhanced monocytopoiesis (7). On days 1 and 2, there were many early granulocytes with irregularly lobulated nuclei in bone marrow. These cells may be monocyte progenitors and represent morphological evidence of enhanced monocytopoiesis (8). Alternatively, these cells may be neutrophil precursors with bizarre nuclear configurations. Attempts to identify these cells with naphthol ASD chloroacetate esterase and α-naphthyl acetate esterase stains were inconclusive. Monocytosis, eosinopenia and lymphopenia can be attri-

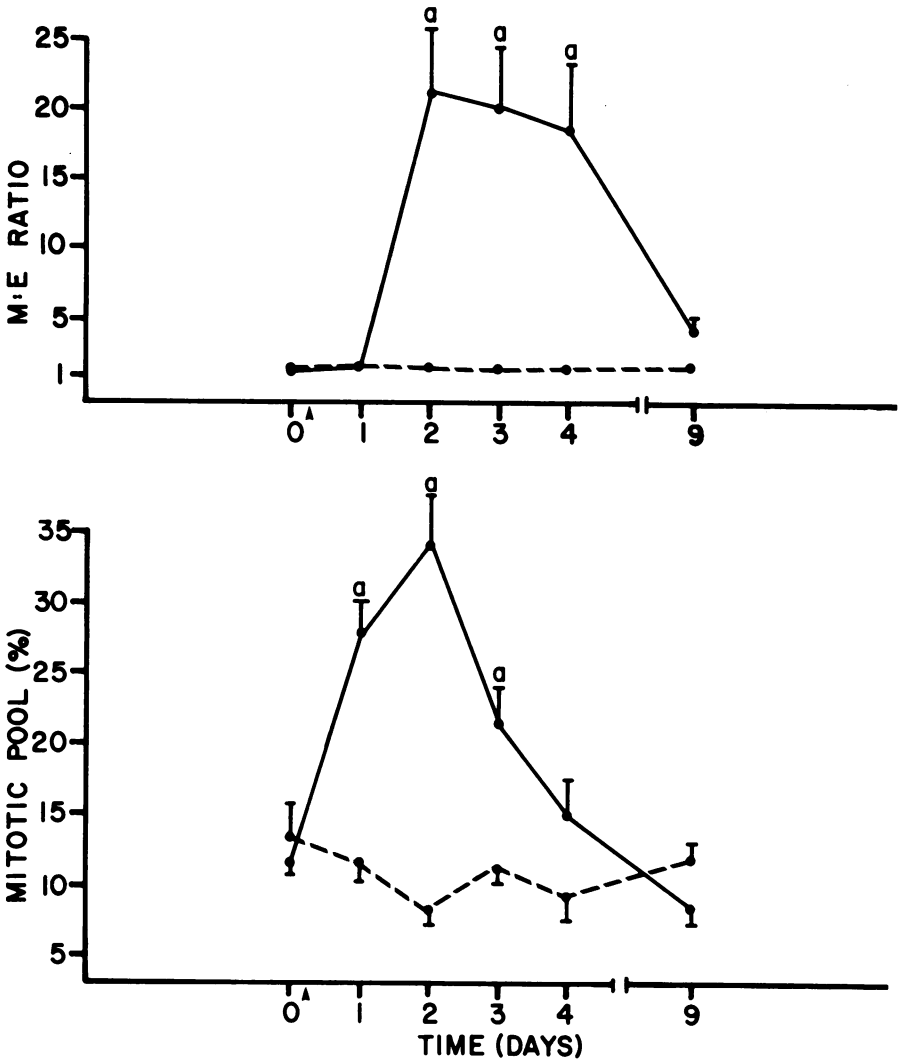

Fig. 5. Myeloid:erythroid ratio and mitotic pool as a percentage of total myeloid mass from bone marrow of control (---) and turpentine-injected dogs (___). Each point represents $\bar{x} \pm$ SEM, n = 4. Arrows signify time of injection.
a = means are significantly different ($P < 0.01$)
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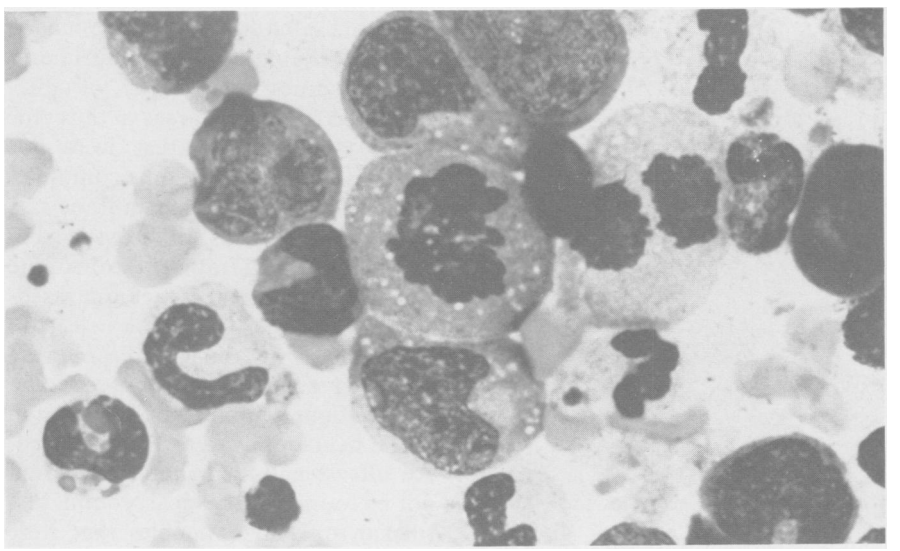

Fig. 6. Canine bone marrow collected 24 hours after turpentine injection. Two neutrophil precursors with cytoplasmic vacuoles are in mitosis. Modified Wright's stain. X1100.

buted at least in part to elevated endogenous glucocorticoid levels. The return of eosinophils to circulation following an eosinopenic episode has been associated with a more favorable prognosis (1). At day 6, abatement of the eosinopenia, clinical signs and pyrexia was accompanied by decreasing numbers of toxic neutrophils. These data suggest that changes in numbers of toxic neutrophils can be used as a prognostic indicator.

Several changes were observed in the bone marrow of dogs given turpentine. On day 1, the relative decrease in the postmitotic pool in conjunction with an unchanged M:E indicates that the neutrophilia during the first 24 hours was due to increased release of segmented and band neutrophils from the bone marrow. The samples on day 1 had increased percentages of myeloblasts and progranulocytes; granulocyte mitotic figures were numerous. The relative increase in the mitotic pool did not produce an increase in the M:E because of concurrent depletion of the postmitotic pool. Differentiation of the mitotic pool with repopulation of the postmitotic compartment resulted in a high M:E on days 2 through 4. The neutrophilic leukocytosis and left shift indicate that the increased M:E was due primarily to increased granulocytic activity. However, a mild but statistically significant decrease in the PCV suggests that decreased erythroid activity also contributed to the increased M:E. Turpentine-induce inflammation has been shown to decrease erythropoiesis in the cat (9).

The time sequence of the quantitative change in blood and bone marrow was similar to that which has been observed in previous kinetic studies (3,4). A six to sevenfold increase in the marrow release of neutrophils occurs within seven hours after induction of inflammation. Increased tritiated thymidine uptake and mitotic rate in granulocyte precursors also occur. These studies indicate that addition of committed stem cells to granulopoiesis, increased mitotic activity and accelerated release of neutrophils from the bone marrow contribute to the granulocytic response to inflammation (3,4).

Marrow release of neutrophils is age related; mature cells are released pre-

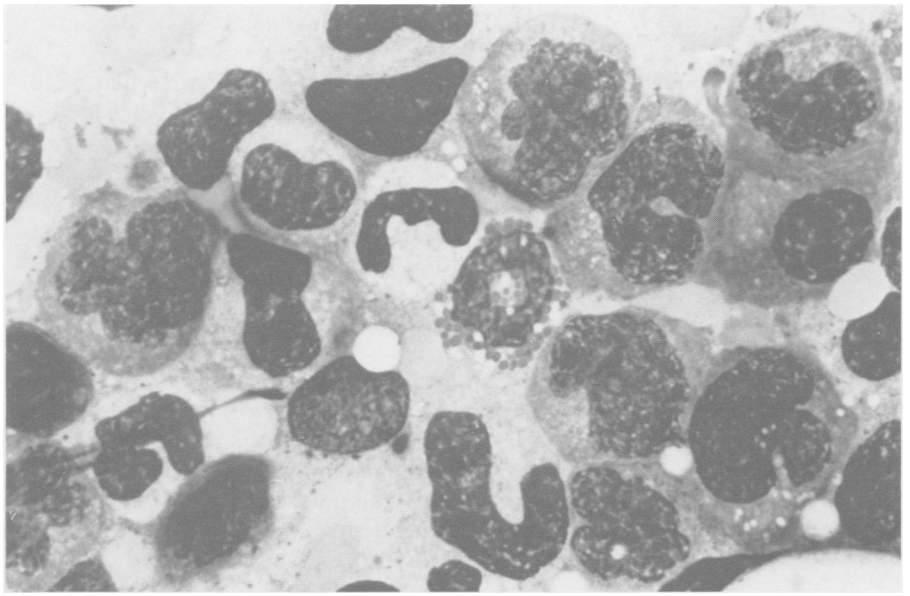

Fig. 7. Canine bone marrow collected 24 hours after turpentine injection. Several progranulocytes have irregular, lobulated nuclei. These cells may be progenitors of monocytes or granulocytes. Modified Wright's stain. X1100.

ferentially in response to increased demand. When the marrow reserve of mature neutrophils is depleted, band neutrophils are released. As granulocytic hyperplasia replenishes the postmitotic pool, the release of mature neutrophils supersedes entry of band neutrophils into circulation and the left shift subsides (10). In our experiment, the abatement of left shift by day 4 corresponded to the intense granulocytic hyperplasia in the bone marrow while neutrophilia persisted through day 6.

Morphological abnormalities in the marrow granulocytic series were observed from day 1 through 4. The severity and character of the marrow lesions progressed with time. The occurrence of clear, round, cytoplasmic vacuoles with sharply defined margins progressed from the myeloblast/progranulocyte stages at day 1 to the myelocyte/metamyelocyte stages by days 2 and 3. Lobulation and blebbing of the nucleus in myeloblasts and progranulocytes were most severe on days 1 and 2. Cytoplasmic basophilia and foaminess were noted in myelocytes, metamyelocytes, bands and segmented neutrophils at days 1 and 2. These nuclear and cytoplasmic alterations have been observed by others in marrow specimens obtained from clinical cases (1,2,11) but this experiment elucidates the progression of the marrow lesion with time and the relationship between the marrow findings and the hematological data. Differences between control and test dogs for M:E, percentage of cells in the postmitotic pool and number of mitotic figures were greatest on days 1 through 3 when the number of toxic neutrophils in circulation were maximal. In addition, significant numbers of toxic neutrophils persisted in circulation for several days after the morphological changes in the marrow had subsided.

In vitro exposure to EDTA potentiates toxic change in canine neutrophils. However, this effect is very mild from a clinical standpoint and should not prevent accurate interpretation of blood smears (12). In the present study, toxic neutrophils in bone marrow which had not been exposed to EDTA and the lack of toxic neutrophils in blood and bone marrow of control dogs further support that toxic change is clinically significant, rather than merely an artifactual change.

Ultrastructural examination of the circulating toxic neutrophils from dogs given turpentine revealed increased amounts of rough endoplasmic reticulum, mitochondria and polyribosomes and irregular, variable-sized electron lucent areas in the cytoplasm (13). Lucent areas were not membrane bound but some contained remnants of a limiting membrane and dense myelin figures. In the bone marrow, dilation and fragmentation of the rough endoplasmic reticulum associated with large, amorphous, electron lucent areas in the cytoplasm were observed in neutrophil precursors. Myelin figures similar to those seen in blood neutrophils were also observed. An ultrastructural counterpart could not be found for the clear, round, distinct cytoplasmic vacuoles that were seen in granulocytes precursors at the light microscopic level (13).

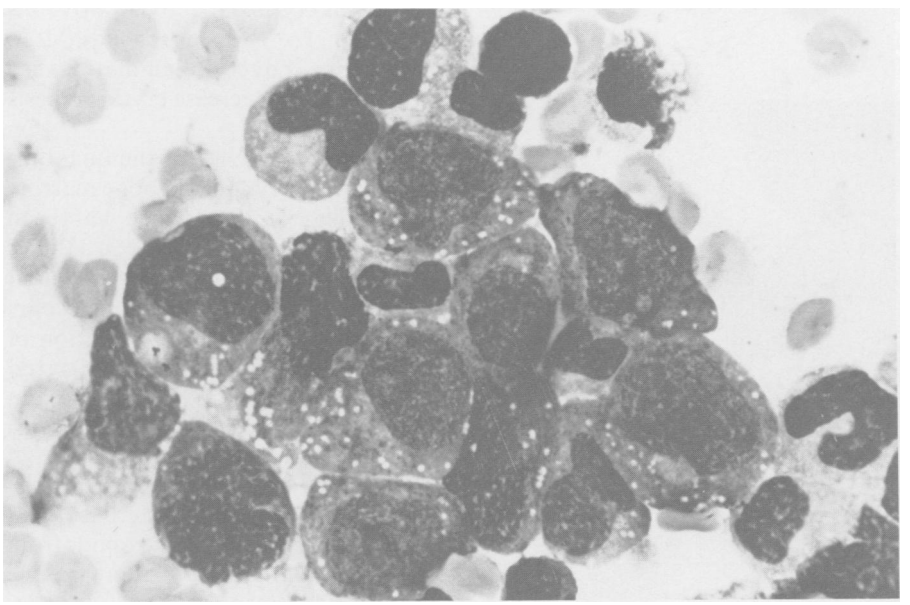

Fig. 8. Canine bone marrow collected 24 hours after turpentine injection. Myeloblasts and progranulocytes contain numerous distinct, clear, intracytoplasmic vacuoles. Modified Wright's stain. X1100.

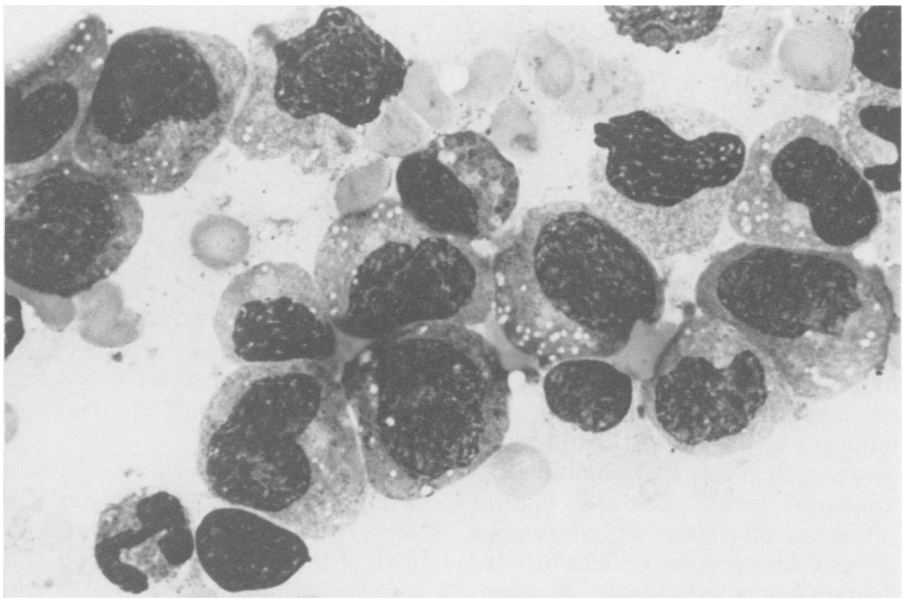

Fig. 9. Canine bone marrow collected 48 hours after turpentine injection. Myelocytes and metamyelocytes contain numerous distinct, clear cytoplasmic vacuoles. Modified Wright's stain. X1100.

The morphogenesis of toxic change in canine neutrophils remains speculative. Ultrastructural studies indicated that immaturity and degeneration of the cytoplasm are involved (13). The appearance of significant numbers of toxic neutrophils at eight hours postinjection, when little new production of neutrophils could have occurred, supports the involvement of cellular degeneration. These degenerative changes may be a result of toxemia and stress of rapid neutrophil production.

If cytoplasmic immaturity is involved, the appearance of toxic neutrophils at eight hours after injection indicates that this change can occur in the final stages of maturation. There was a lack of correlation between number of band neutrophils and number of toxic neutrophils and an apparent lack of correlation between severity of toxicity and nuclear immaturity. This suggests that if toxic change is due to cytoplasmic immaturity, there is asynchrony of nuclear and cytoplasmic maturation.

The time sequence of morphological and quantitative changes in bone marrow neutrophils in the present study were similar to the results of previous kinetic studies (3,4). Depletion of the bone marrow storage pool of mature neutrophils and an increase in mitotic activity and number of early granulocyte precursors were evident within 24 hours. During the next three days, intense granulocytic hyperplasia resulted in replenishment of the bone marrow storage pool. Addition of stem cells to granulopoiesis, increased mitotic activity and accelerated release of neutrophils from bone marrow all amplify the granulocytic response to inflammation in the dog (3).

Toxic neutrophils were present in the blood by eight hours postinjection. The number of toxic neutrophils paralleled the intensity of clinical signs and changes in rectal temperature but not the number of band neutrophils. This indicates that the number of toxic neutrophils is a helpful prognostic indicator in dogs with severe localized inflammation.

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