Serum Colony-stimulating Activity of Dogs With Cyclic Neutropenia

By T. J. Yang, J. B. Jones, E. S. Jones, and R. D. Lange

Bone marrow cells, from both normal and cyclically neutropenic (CN) dogs, treated with CN or normal dog serum produced large numbers of leukocytic colonies in soft agar cultures. The largest number of colonies was formed in cultures treated with serum taken from CN dogs at the nadir of the neutropenia. The serum colony-stimulating activity was shown to be inversely related to the CN dog’s peripheral neutrophil counts, suggesting a negative feedback control of granulopoiesis.

Canine cyclic neutropenia (CCN) is characterized by a profound and cyclic decrease in circulating granulocytes at 11–12-day intervals. In addition, the marrow of cyclically neutropenic (CN) dogs has been shown to have synchronous cycles of neutrophil maturation. During the neutropenic episodes, the marrow of CN dogs is characterized by large blastlike cells capable of differentiating into granulocytes, while 6–8 days later the CN dog’s marrow shows evidence of an arrest in granulocyte maturation. Using the recently developed soft agar culture system, which supports the colony growth of granulocytes and macrophages, it is now possible to undertake more detailed studies of the regulatory mechanism of granulopoiesis.

It is the purpose of this report to describe the in vitro leukocyte colony responses of normal and CN dog marrow to the sera of normal and cyclically neutropenic dogs throughout the cycles of neutropenia.

MATERIALS AND METHODS

Dogs

Serum donors. Five CN dogs (CN 172, 180, 181, 187, 188), an adult mongrel (No. 4), and one normal collie male (No. 214) whose parents had been tested free of the CN gene were used as serum donors. CN dogs 187 and 188 were female litter mates 11–15 wk of age; CN-180, a female, and CN-181, a male, were litter mates. All dogs whose sera were used in these studies were bled from the jugular vein between 8:30 and 9:00 a.m. each day and the leukocyte counts determined with an autocytometer II (Fisher Scientific). Serum samples were obtained daily from CN dogs throughout one to two 12-day neutropenic cycles and randomly at other times. Samples were stored at –20°C until tested. Serum samples from normal dogs were similarly processed. Wright’s stain was used for preparation of smears for differential leukocyte counting.
Marrow donors. Seven young adult mongrel dogs purchased from municipal pounds and one CN dog (CN-187) were used as marrow donors. Following exsanguination under pentobarbital anesthesia, the femurs of CN and normal dogs were removed. The femurs of CN-187 were removed during the neutropenic phase of the disease.

The marrow was collected aseptically in T.C. Hanks' solution (Difco Laboratories, Detroit, Mich.) from the bones, broken at the midpoint of the shank. The marrow was aspirated and flushed through an 18-gauge needle. The flushings were pipetted repeatedly to disperse the cells and then placed upright in test tubes for 10 min. The floating fat layer which contained very few cells was then discarded, and the remaining portions were pooled. The number of red cells present in the preparations was small. Viable cells were counted by the trypan blue dye exclusion test. The cells which were larger than those of the mature leukocytes were designated as viable bone marrow cells. This designation was made because our preliminary studies and those of Moore et al. have shown that only viable cells approx 9 μ or larger will form in vitro colonies in soft agar.

Soft Agar Culture

A modification of the methods of Metcalf and Moore, Levitt et al., and Yang and Vas is described as follows: Double-strength Medium 199 (Grand Island Biological Co., Grand Island, N.Y.) supplemented with heat-inactivated, virus-screened, fetal calf serum (Grand Island Biological Co.), penicillin, and streptomycin was sterilized with sintered glass filters and stored at -20°C until use. The same batch of the medium was used throughout an experiment. For preparation of soft agar cultures, aliquots of all suspensions containing 10 x 10⁶ viable marrow cells were centrifuged, the cell pellets resuspended into 5 ml of double-strength Medium 199 (kept at 37°C) and then mixed with an equal volume of 0.6% purified agar (Difco Laboratories) in distilled water (kept at 45°C) to make the final concentration of 0.3% agar, 10% fetal calf serum, 100 U of penicillin, 100 μg streptomycin, and 1 x 10⁶ viable marrow cells per ml of medium. The cell suspension (1.2 ml) in soft agar medium was poured into 35 x 10-mm plastic Petri dishes (Falcon 3001, Falcon Plastics, Oxnard, Calif.) and allowed to harden. After the agar was solidified, 0.2 ml of test serum was spread over the top of the agar. The plates were incubated in a humidified atmosphere of 5% CO₂ in air at 37°C. After 7-9 days, colonies were counted by use of an inverted microscope. Morphologically, three distinct colony types, (compact, mixed, and loose) were observed, but no special effort was made to type each colony for accurate colony classification. As suggested by Marsh et al., the results were expressed simply as the number of colonies of granulocytic-monocytic series. Four Petri dishes were counted for each serum tested, and the results are given as the mean ± SEM of the number of colonies per dish. A colony was defined as a group of more than 20 cells. The justification in regarding the number of colonies formed at 7-9 days and of 20 cells as composing a colony was based on the findings of Marsh et al. They showed that the dog bone marrow colonies formed early, at 7-11 days, were composed predominately of peroxidase-positive cells, many of which had horseshoe shaped and segmented nuclei consistent with neutrophilic granulocytes, while those formed at 12-18 days were predominately of mononuclear cells.

In all experiments two sets of control cultures were included: (1) culture media alone, and (2) culture media plus serum of normal dog No. 4. These controls allowed the test sera's stimulating effects to be compared to the basic media and to normal sera for each marrow target population.

RESULTS

In Table 1 are presented, from several experiments, the mean number of colonies formed in soft agar medium treated with (1) no serum, (2) normal dog No. 4 serum, and (3) serum from CN dogs experiencing severe neutropenia. The data show that normal dog serum stimulated colony formation, and the neutropenic serum from the CN dog produced an even greater stimulation. The same relationship of stimulation (medium controls compared to test sera) prevailed throughout a series of experiments assaying the effects of daily serum...
samples of CN dogs on soft agar cultures of canine marrow. In each experiment, the absolute number of colonies varied, since normal target marrow came from a different donor for each experiment.

The neutrophil counts for each day of the cycle (CN 180 and 188) are shown in Fig. 1. In this same figure are shown the numbers of granulocyte-monocyte colonies that formed in soft agar when normal marrow cells were treated with sera collected on the same day that neutrophil counts were made. When neutrophil counts were lowest, the numbers of leukocyte colonies formed in soft agar were at their peak. Although not shown, serum from CN 172, 181, and 187 produced similar responses when studied over one and two cycles.

Normal canine marrow, plated and then treated with serum samples from a dog free of the CN gene (No. 214), showed, during a 12-day period, a uniform colony-stimulating activity (CSA) (Fig. 2).

Table 1. Colony Formation* in Soft Agar Cultures of Dog Marrows Treated With Various Sera

<table>
<thead>
<tr>
<th>Marrow Donor (Target Cell)</th>
<th>Medium Control</th>
<th>Normal Dog Serum No. 4</th>
<th>Neutropenic Serum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal dog A</td>
<td>33.7 ± 4.3</td>
<td>127.3 ± 13.1</td>
<td>404.0 ± 24.9</td>
</tr>
<tr>
<td>Normal dog B</td>
<td>16.0 ± 0.6</td>
<td>143.6 ± 3.9</td>
<td>248.5 ± 25.6</td>
</tr>
<tr>
<td>Normal dog C</td>
<td>0.0</td>
<td>147.9 ± 7.0</td>
<td>225.9 ± 6.6</td>
</tr>
<tr>
<td>Normal dog D</td>
<td>10.3 ± 2.0</td>
<td>112.7 ± 2.8</td>
<td>173.2 ± 6.6</td>
</tr>
<tr>
<td>Normal dog E</td>
<td>10.3 ± 0.7</td>
<td>49.8 ± 4.6</td>
<td>217.2 ± 16.3</td>
</tr>
<tr>
<td>CN 187</td>
<td>38.1 ± 0.9</td>
<td>221.5 ± 17.4</td>
<td>347.6 ± 5.8</td>
</tr>
</tbody>
</table>

*Mean colony count ± SEM per Petri dish. 1.2 x 10^6 marrow cells were plated into each dish.
†Serum collected at nadir of neutropenia during 12 daily collections.

Fig. 1. Relationship between daily neutrophil counts of CN dogs and the number of leukocytic colonies formed in soft agar cultures of normal marrow cells treated with CN sera. (A), CN-187; (B), CN-180.
The marrow of CN dog 187, collected at the neutropenic phase of the cycle, responded to CN sera 188 by forming large numbers of colonies in soft agar (Fig. 3). By comparing Figs. 1 and 3, it is readily apparent that the pattern of colony formation is very similar for normal and CN marrow.

The suggested inverse relationship (Figs. 1 and 3) of peripheral neutrophil counts to serum CSA is more obvious when further statistical calculations are made. In Fig. 4 is shown the plot of the log of the number of soft agar colonies against the log of the peripheral neutrophil count, using figures from three experiments in which normal marrow target cells were treated with CN sera. The linear correlation coefficient ($R$) was $-0.627$, which was statistically significant at the 0.1% level ($p < 0.001$). The same relationship was shown to occur when CN marrow target cells are treated with CN sera (linear correlation coefficient $R = -0.577; p < 0.01$). In Table 2, the comparable results from seven experiments are shown. It should be pointed out that the inverse relationship is most obvious when the neutrophil count is undergoing regular changes.
Fig. 4. Correlation between peripheral blood neutrophil count and serum soft agar colony stimulating activity of CN-dog 187. Marrow cells from three normal dogs were used as target cells in soft agar colony assay in three experiments. Each point represents the determination from a 1-day sample from three 11-day neutropenic cycles of CN-dog 187. \( r = -0.627; p < 0.001; Y = 3.084 - 0.243 X. \)

(Fig. 1A) and the results given in Fig. 3 and Table 2 are based on data from CN dogs 187 and 188.

There is little doubt that the sera of CN dogs contains a factor capable of stimulating soft agar colony growth and that the factor is most active when the CN dogs are experiencing profound neutropenia. In the study of sera collected during 14 complete or partial cycles of neutropenia, the highest number of

<table>
<thead>
<tr>
<th>Marrow Donor (Target Cell)</th>
<th>Serum Donor</th>
<th>No. of Daily Samples</th>
<th>Correlation Coefficient ( (r) )</th>
<th>Significance ( (p) )</th>
<th>Linear Regression Line ( Y = B - AX )</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN-187 marrow</td>
<td>CN-187</td>
<td>11</td>
<td>-0.653</td>
<td>&lt;0.05</td>
<td>( Y = 2.991 - 0.214X )</td>
</tr>
<tr>
<td>CN-187 marrow</td>
<td>CN-188</td>
<td>11</td>
<td>-0.803</td>
<td>&lt;0.01</td>
<td>( Y = 3.308 - 0.255X )</td>
</tr>
<tr>
<td>Normal dog A</td>
<td>CN-187</td>
<td>11</td>
<td>-0.767</td>
<td>&lt;0.01</td>
<td>( Y = 4.161 - 0.546X )</td>
</tr>
<tr>
<td>Normal dog B</td>
<td>CN-187</td>
<td>12</td>
<td>-0.683</td>
<td>&lt;0.01</td>
<td>( Y = 3.019 - 0.213X )</td>
</tr>
<tr>
<td>Normal dog C</td>
<td>CN-187</td>
<td>12</td>
<td>-0.569</td>
<td>&lt;0.05</td>
<td>( Y = 2.622 - 0.126X )</td>
</tr>
<tr>
<td>Normal dog C</td>
<td>CN-188</td>
<td>12</td>
<td>-0.414</td>
<td>&lt;0.1</td>
<td>( Y = 2.767 - 0.153X )</td>
</tr>
<tr>
<td>Normal dog D</td>
<td>CN-188</td>
<td>12</td>
<td>-0.735</td>
<td>&lt;0.01</td>
<td>( Y = 3.025 - 0.288X )</td>
</tr>
</tbody>
</table>
colonies observed in an experiment always occurred at or just before the onset of neutropenia.

**DISCUSSION**

In 1966, a soft agar culture technique which supports the colony growth of granulocytes and macrophages was developed independently by Bradley et al. and Ichikawa et al. This technique has made it possible to undertake detailed studies on the existence and nature of possible humoral regulators of granulopoiesis. A recent finding of a colony-stimulating factor (CSF) in most normal sera, including dogs, and a greatly enhanced activity of the factor in leukemic AKR mice strongly suggests that cyclic fluctuation of the factor would be expected in CN dogs which exhibit a marked cycle of granulopoiesis. In fact, Dale et al. found that the urine of CN dogs contained CSA for the mouse marrow system and that the level of CSA reached a peak at or near the periods of neutropenia. In the present experiments the canine bone marrow system was used for the assay of CSA in serum samples taken from CN dogs throughout the neutropenic cycle. Marsh et al. have previously shown that the canine bone marrow system is useful for assaying canine serum for CSA. With the use of the homologous system, problems of interpretation which arise in heterologous systems can be avoided; e.g., in the mouse marrow system, normal human serum is less active, due to strong inhibiting activity.

As shown in Table 1, the number of colonies formed in the absence of either normal or CCN sera (i.e., medium control) was much smaller than when sera were added. This indicated that possible autostimulation due to high number cell inoculation (1.2 x 10^6 per dish) did not contribute to the differences observed. High inoculum was used in order to minimize the counting errors that are inherent in small numbers. The 1.2 x 10^6 plated marrow cells were larger than 9 μ, and all mature nucleated cells were excluded from enumeration. The marrow preparations were obtained from exsanguinated dogs and contained only 10% mature cells. The absence of significant quantities of peripheral cells in the plating marrow preparations produced culture plates with low backgrounds of nonproliferating cells. Although colonies were defined as having 20 or more cells, many colonies resulting from CN serum stimulation consisted of several hundred cells. That the efficiency of colony formation with sera can vary with cells from different bone marrows is well known and was apparent in our various experiments. However, in all experiments, comparison of controls to test sera indicated that the number of colonies stimulated by serum collected during the neutropenia reflected true increase in CSA rather than relative concentration of cells with growth potential.

In this report, we have shown that dogs with cyclic neutropenia have a factor(s) in their serum capable of stimulating leukocytic colony growth in soft agar cultures of both normal marrow and CN marrow. The activity in the CN serum fluctuates cyclically in a manner inversely related to the peripheral neutrophil count. This finding suggests that a negative feedback regulation of granulopoiesis exists in vivo. Further control studies showed that normal canine sera stimulated normal canine marrow to form colonies, but not in a
cyclic manner. Therefore, it is clear that the level of serum leukocytic colony-growth factors cycles in CN dogs. For the first time, it is possible to measure a regularly recurring relationship between granulocytic colony growth in soft agar and the circulating neutrophil levels. In considering the implications of a negative feedback control mechanism of granulopoiesis, it should be pointed out that there is evidence from a human study that a positive feedback control is operative in some cases. However, a recent report supports the double feedback theory as proposed by Morley et al.

Significant changes in the CSA of daily serum samples taken from CN dogs suggest that the half-life in vivo is rather short. This is in agreement with the report by Metcalf et al. that serum half-life of CSF is less than 3 hr. Furthermore, CSF has been shown to have a concentration-dependent action in stimulating colony formation. In our studies, a rather high number of marrow cells was plated. However, the ratio of marrow cells to serum \((10^6 \text{ per } 0.2 \text{ ml})\) was similar to the optimum ratio found by Morley et al.

A complex of lipoprotein “CSF inhibitors,” which prematurely transforms colonies to macrophages, has been found to be present in high concentrations in normal human sera. Furthermore, the presence of an inhibitory hormone for hematopoiesis or “chalone” has been proposed, and it is possible that an intermittent excess of the inhibitor or blocking agent present in CN sera at certain phases of the cycle may be preventing the granulocytic progenitors from responding to granulopoietic factors. This possibility was not directly explored. It is entirely possible that several factors affecting granulopoiesis are present in the sera of CN dogs used in the CN studies, since no attempts were made to separate serum components. Preliminary studies have indicated that heat inactivation (56°C, 30 min) of the serum causes slower growth of the cells, resulting in smaller colonies. Whether this is due to depletion or decrease in the concentration of a factor remains to be elucidated.

CN dogs experiencing a well-defined, wide range of circulating peripheral neutrophils showed an inverse relationship between soft agar colony number and the neutrophil count (Figs. 1A, 3, and 4). However, the pathogenesis of canine CN is not fully understood. During some, but not all cycles, infections, very high rebound neutrophilias, and variations in the severity of the neutropenia occur (Fig. 1B). These factors may affect experimental results. Since significant levels of CSF have been detected in the serum from mice treated with endotoxin, the possible role of external factors such as infection and endotoxin on the CSA of CCN sera was considered. However, available data show that the cyclic peaks in CSA seemed not to depend on fever or other signs of infections such as high rebound neutrophilia (Fig. 1B). In studying sera from 14 neutropenic cycles, the serum CSA was always greatest during the neutropenia. Signs of infection (elevated body temperature, rebound neutrophilia, etc.) were not present during six of the cycles. Therefore, endotoxin from infections may have affected CSA (Fig. 1B), but it did not change the cyclic peak of serum CSA.

We have so far studied only the effects of CN serum on the bone marrows from seven normal dogs and the response of the bone marrow of a CN dog...
taken during peripheral blood neutropenic phase. The study of the CN marrow at other stages of the disease must await the availability of more dogs affected with CN.

REFERENCES

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