Inhibition of CFU-NM and CFU-EOS by Mature Granulocytes

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Approximately half of the colony-forming units-culture (CFU-C) from normal peripheral blood are eosinophilic. The purpose of our study was to determine: (1) whether progenitor cells committed to eosinophil or neutrophil maturation would be differentially affected by feedback inhibition, and (2) whether mature eosinophils added to the feeder layers of the culture would inhibit the proliferation of CFU-C in a manner similar to that described for neutrophils. Concentrated eosinophils and neutrophils, obtained by separation on a metrizamide gradient, were added to feeder layers containing either 10^6 autologous whole mononuclear cells (WMNC) or 0.1 ml of leukocyte conditioned media (LCM). The average number of colonies was 123/10^6 nonadherent cells (NAC) cultured. When neutrophils or eosinophils were added to the WMNC feeder layer, the percent inhibition of growth was 40.2% ± 1.6% (mean ± SEM) and 42.3% ± 5.4%, respectively, but the ratio of neutrophil to eosinophil colonies remained constant. No effect was seen when neutrophils or eosinophils were added to an LCM feeder layer. Thus, it appears that the differential control of neutrophil versus eosinophil production in vitro is not regulated through feedback inhibition by mature granulocytes. In addition, these studies suggest that eosinophils, as well as neutrophils, cause inhibition of CFU-C growth when intact cells are the source of colony-stimulating factor (CSF).

POLYMORPHONUCLEAR neutrophils (PMN) inhibit growth of colony-forming units-culture (CFU-C) by decreasing production of CSF by monocytes.[1] In studies of granulocyte-derived colony-inhibiting activity, growth was reduced to 35%–75% of control, with maximal inhibition at 10^4 PMN. We have found that 49% ± 8% (n = 25) of the CFU-C cultured from peripheral nonadherent cells (NAC) are eosinophilic.[2] As the earlier studies of granulocyte inhibition did not distinguish between neutrophil/monocyte colonies (CFU-NM) and eosinophil colonies (CFU-EOS),[1,3] we undertook this study to determine whether feedback inhibition modifies the ratio of CFU-EOS to CFU-NM. In addition, we examined the effect of peripheral blood eosinophils on the growth of CFU-EOS and CFU-NM.

The present experiments show that, although neutrophils inhibit CSF production, the ratio of CFU-EOS to CFU-NM is unaffected by this inhibition. They further suggest that mature eosinophils may also inhibit in vitro granulopoiesis.

MATERIALS AND METHODS

Peripheral blood was collected in preservative-free heparin from six healthy volunteers (four males and two females) and one patient with the hypereosinophilic syndrome[4] and a peripheral eosinophil count of 92,000/cu mm.

Preparation of Cells

Whole mononuclear cells (WMNC) were separated from whole blood using Ficoll-Hypaque.[5] After washing the WMNC 3 times in alpha-minimal essential medium (alpha-MEM), a portion of WMNC was saved for use in the feeder layer. The remainder was suspended in 5 ml of alpha-MEM containing 20% fetal calf serum (FCS) and incubated in plastic Petri dishes at 37°C for 1 hr to remove adherent monocytes, which are a source of colony-stimulating factor (CSF).[6] The nonadherent cells (NAC) were collected from the supernatant and incubated in fresh Petri dishes for another hour.

Eosinophil and Neutrophil Concentrations

A 7-step metrizamide gradient was prepared in 15-ml conical plastic tubes, with a minor modification of a previously described technique.[7] Metrizamide solutions of 18%, 20%, 22%, 23%, 24%, 25%, and 26% were made by diluting 30% metrizamide with Tyrodes gel/DNase. One milliliter of PBS, containing 7 × 10^7 buffy coat cells prepared by dextran sedimentation, was layered on the preformed gradient. After centrifugation at 1,200 g for 45 min at 22°C, cells were covered from each interface, counted, and washed. Erythrocytes were removed by hypotonic lysis. Preparations used contained greater than 72% eosinophils and greater than 85% neutrophils, except for the hypereosinophilic syndrome patient sample, which was 100% eosinophils.

Preparation of Leukocyte Conditioned Media

A modification of the method described by Iscove et al.[8] was employed to prepare leukocyte conditioned media (LCM). Twenty million WMNC, obtained from a patient with idiopathic leukopenia, were incubated for 7 days at 37°C in 5% CO_2 in alpha-MEM (Eagle-modified) containing 15% FCS. At the end of 14 days, the medium was centrifuged at 1,200 g for 15 min. The supernatant was stored at 4°C until use. Titration revealed maximal colony-stimulating activity with 0.1 ml LCM/1 ml feeder layer.

Preparation of Cultures

A modification of the double-layer soft agar technique of Pike and Robinson was used.[9] The overlayer in all experiments contained 10^6 NAC in 1 ml of alpha-MEM, 15% FCS, and 0.3% agar. The feeder layer contained either 10^6 WMNC or 0.1 ml of LCM in alpha-MEM to a final volume of 1 ml, containing 15% FCS and 0.5% agar.

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Presented in part at the Cellular and Molecular Biology of Hemopoietic Stem Cell Differentiation Meeting, Ontario, Canada, September 1981.

Supported in part by NIH Grants Hematology Training HL 07437-04 HL 15157-10, and AI 16432. B.H.B. is supported by the Medical Foundation: Charles A. King Research Fellowship. S.H.P. was supported by an NIH Clinical Investigator Award K08 AM 00538.

Submitted April 1, 1983; accepted August 24, 1983.

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0006-4971/84/6302-0019$01.00/0

Table 1. Differential on the Granulocyte Concentrations Added to the Feeder Layer

Experiment No.	Eosinophil Concentrate (Pellet)	Neutrophil Concentrate (Fraction 24)
1	28/72/0*	98/1/1*
2	17/83/0	87/9/4
3	20/80/0	90/9/1
4	25/75/0	85/15/0
5	5/95/0	95/3/1
6	0/100/0	—

*Proportions of neutrophils, eosinophils, and monocytes in the preparations added to the feeder layers.

Eosinophils or neutrophils (2×10^3, 2×10^4, and 2×10^5) were added to feeder layers containing either WMNC or LCM. These particular concentrations were selected because previous studies had shown that maximal inhibition was achieved at 10^4 PMN/1 ml of feeder layer.[1] The cultures were incubated for 14 days at 37°C in a humidified 5% CO_2 atmosphere. All cultures were prepared in triplicate.

Staining and Scoring

After 14-day incubation, the double-layer agar disc was floated in water. Overlayers were separated from feeder layers by gently teasing them apart with a metal spatula. The thin overlayer was floated onto a 75 × 50 mm glass slide and allowed to dry overnight.

The disc was stained with 0.1% Luxol Fast Blue MBS for 2 hr, rinsed in running tap water for 2 hr, and counterstained with hematoxylin for 2 min. Colonies, defined as discrete collections of 40 or more cells, were scored with a light microscope at 100× magnification. Eosinophil colonies were easily distinguished from neutrophils colonies by their bright aquamarine granules and their usually tight distribution of cells. In contrast neutrophil/macrophage colonies stained a pale gray blue and tended to grow in a more loose pattern. Results are presented as the mean of triplicate plates.

RESULTS

Neutrophils obtained from fraction 24 and eosinophils obtained from the pellet were used in all experiments. The purity of these preparations is indicated in Table 1. The percentage of eosinophils ranged from 72% to 100%, the remaining cells being neutrophils.

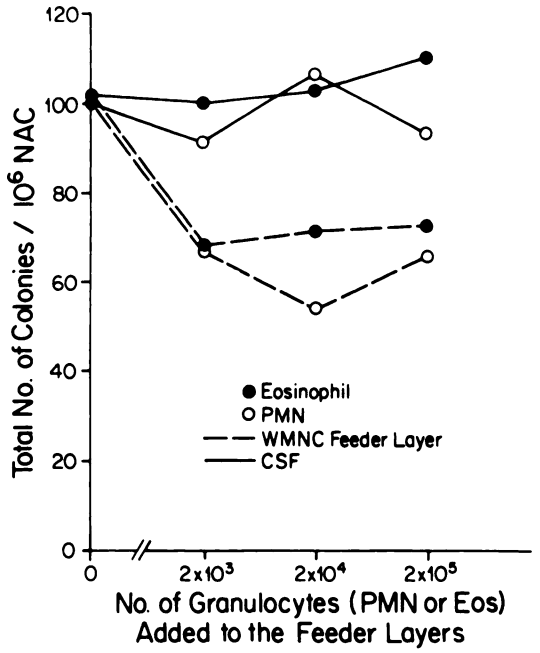

Fig. 1. Inhibition of CFU-C production by granulocytes. The number of eosinophils (closed circles) or neutrophils (open circles) added to feeder layers containing WMNC (dashed line) or preformed CSF (solid line) were varied as indicated. Results are expressed as the total number of colonies per 10^6 nonadherent cells (NAC) cultured and represent the mean of triplicate values from experiment 4.

The percentage of neutrophils was 85%–98%, the remainder being mainly eosinophils.

Inhibition was present when granulocytes were added to WMNC. The degree of inhibition was equivalent when eosinophil or neutrophil concentrations were employed. Figure 1 expresses the results from experiment 4. The degree of inhibition did not vary with the number of neutrophils or eosinophils added to the feeder layer.

Similar findings were present in all five experiments. The percent inhibition with either 2×10^5

Table 2. Percent Inhibition of Colony Growth by Eosinophils and Neutrophils With LCM or WMNC Feeder Layers

	Feeder Layer					
	WMNC			LCM		
Exp. No.	Alone (Total Colonies)	± Eosinophils % Inhibition*	± Neutrophils % Inhibition*	Alone (Total Colonies)	± Eosinophils % Inhibition*	± Neutrophils % Inhibition*
1	68	56%	41%	—	—	—
2	222	30%	46%	—	—	—
3	126	31%	40%	—	—	—
4	100	31%	38%	105	4%	15%
5	161	48%	36%	125	3%	5%
6	60	58%†	—	51	8%†	—
Mean ± SEM	100%	42.3% ± 5.4%	40.2% ± 1.7%		5% ± 1.5%	10% ± 5%

*Results are expressed as the percent inhibition: [100 − (Experimental/Control)] × 100, and represent the mean of triplicate values.

†Eosinophil preparations obtained from hypereosinophilic syndrome patient contained 100% eosinophils.

$p < 0.0005$ versus WMNC alone.

$p < 0.0125$ versus LCM alone.

$p < 0.025$ versus LCM alone.

Table 3. Percent Eosinophil Colonies: Effect of Addition of Granulocytes to LCM or WMNC Feeder Layers

	Feeder Layer					
	WMNC			LCM		
Exp. No.	WMNC Alone (% Eos Colonies)	WMNC ± EOS (% EOS Colonies)	WMNC ± Neut. (% EOS Colonies)	LCM Alone (% EOS Colonies)	LCM ± EOS (% EOS Colonies)	LCM ± Neut. (% EOS Colonies)
1	53%	55%	57%	—	—	—
2	46%	52%	54%	—	—	—
3	50%	50%	48%	—	—	—
4	35%	32%	30%	56	55	54
5	58%	57%	63%	63	60	64
6	25%	34%	—	30%	33%	—
Mean ± SEM	44.5% ± 5.0%	46.7% ± 4.4%*	50.4% ± 5.6%	49.7% ± 10.0%	49.3% ± 8.3%	59% ± 5.0%

*$p < 0.35$ versus WMNC alone.
$p < 0.45$ versus WMNC alone.
$p < 0.15$ versus LCM alone.
$p < 0.40$ versus LCM alone.

neutrophils or 2×10^5 eosinophils in a WMNC feeder layer ranged from 30% to 58% (Table 2). In contrast, no effect was seen when neutrophils or eosinophils were added to a feeder layer containing LCM (Table 3). The ratio of eosinophilic to neutrophilic colonies was not influenced by the presence of peripheral neutrophils or eosinophils in either type of feeder layer (Table 3).

DISCUSSION

Earlier reports on the inhibition of in vitro granulopoiesis by mature granulocytes did not distinguish neutrophil from eosinophil colonies.[1,3] More recently, Pelus et al. demonstrated that addition of iron-saturated lactoferrin inhibits both types of colonies to the same degree.[10] Our experiments demonstrate that the ratio of CFU-EOS to CFU-NM is not affected by the presence of either eosinophilic of neutrophilic granulocytes in the feeder layer. Murine studies suggest that neutrophil CSF may be distinct from eosinophil CSF,[11] and preliminary work with human cells also supports the concept of distinct types of CSF. Nicola fractionated human placental conditioned media into two substances. One was capable of stimulating only CFU-NM and the other of stimulating both CFU-EOS and CFU-NM.[12] Feeder layers containing WMNC produce CSF,[13] which stimulates both eosinophil and neutrophil maturation.[2]

In our experiments, peripheral blood eosinophils added to a feeder layer containing intact monocytes decreased CFU-C growth. Since no effect was seen when eosinophils were added to a feeder layer containing LCM, the mechanism appears to require the presence of intact monocytes and is analogous to that previously described for inhibition by neutrophils. The effect of eosinophils and neutrophils was equivalent. This is of particular interest, as it has been suggested that lactoferrin derived from the secondary granules of neutrophils may be the source of granulocyte colony-inhibiting activity.[14,15] However, eosinophils do not contain lactoferrin,[16] and yet they appear to be equally effective in our system.

It is difficult to obtain pure eosinophil preparations from healthy donors, and it is possible that the inhibition observed when eosinophil concentrates were added in experiments 1–5 was in fact due to contaminating neutrophils. To address this possibility, we established cultures incorporating pure eosinophil preparations from an HES patient (experiment 6). They contained no contaminating neutrophils and yet resulted in significant inhibition. These studies show that, in vitro, the differential production of neutrophils versus eosinophils is not regulated by feedback inhibition. They further suggest that eosinophils are capable of inhibiting granulocyte colony growth.

REFERENCES

1. Broxmeyer HE, Moore MAS, Ralph P: Cell free granulocyte colony inhibiting activity derived from human PMN. Experimental Hematology 5:87, 1977

2. Bjornson BH, Andre-Schwartz J, Desforges JF: In vitro culture of circulating CFU-eos from normal donors. Exp Hematol 10:267, 1982

3. Zucal JR, Broxmeyer HE, Ulatowski JA: Specificity of lactoferrin as an inhibitor of granulocyte-macrophage colony stimulating activity production from fetal mouse liver cess. Blood 54:951, 1979

4. Fauci AS, Harley JB, Roberts WC, Ferrans VJ, Gralnick HR, Bjornson BH: The idiopathic hypereosinophilic syndrome: Clinical, pathophysiologic and therapeutic considerations. Ann Intern Med 97:78, 1982

5. Boyum A: Separation of leukocytes from blood and bone marrow. Scand J Clin Lab Invest 21 (Suppl 97):1, 1968

6. Brennan JK, Lichtman MA, DiPersio JF, Abbaoud CN: Chemical mediators of granulopoiesis: A review. Exp Hematol 8:441, 1980

7. Vadas MA, David JR, Butterworth A, Pisano NT, Siongok TA: A new method for the purification of human eosinophils and neutrophils and a comparison of the ability of these cells to damage *Schistosomula mansoni*. J Immunol 122:1228, 1979

8. Iscove NN, Senn JS, Till JE, McCullough EA: Colony formation by normal and leukemic human marrow cells in culture. Effect of conditioned medium from human leukocytes. Blood 37:1, 1971

9. Pike B, Robinson WA: Human bone marrow colony growth in agar gel. J Cell Physiol 76:77, 1970

10. Pelus LM, Broxmeyer HE, Moore MAS: Regulation of human myelopoiesis by prostaglandin E and lactoferrin. Cell Tissue Kinet 14:515, 1981

11. Ruscetti FW, Cypress RH, Chervenick PA: Specific release of neutrophilic and eosinophilic-stimulating factors from sensitized lymphocytes. Blood 47:757, 1976

12. Nicola NA, Metcalf D, Johnson GR, Burgess AW: Separation of functionally distinct human granulocyte macrophage colony stimulating factors. Blood 54:614, 1979

13. Messner HA, Till JE, McCulloch EA: Interacting cell populations affecting granulopoietic colony formation by normal and leukemic bone marrow cells. Blood 42:701, 1970

14. Broxmeyer HE, Smithyman A, Eger RR, Meyers PA, deSouza M: Identification of lactoferrin as the granulocyte-derived inhibitor of colony-stimulating activity production. J Exp Med 148:1052, 1978

15. Bagby GC, Vasiliki DR, Bennett RM, Vandenbark AA, Garewal HS: Interaction of lactoferrin, monocytes and T lymphocyte subsets in the regulation of steady-state granulopoiesis in vitro. J Clin Invest 68:56, 1981

16. Olsson I, Venge P, Spitznagel JK, Lehrer RI: Arginine-rich cationic proteins of human eosinophil granules. Comparison of the constituents of eosinophilic and neutrophilic leukocytes. Lab Invest 36:493, 1977