References and Notes

1. L. H. Adams and E. D. Williamson, *J. Franklin Inst.* **195**, 475 (1923).
2. J. B. Walsh and W. F. Brace, *Felsmechanik Ingenieurgeol.* **4**, 283 (1966).
3. J. B. Walsh and E. R. Decker, *J. Geophys. Res.* **71**, 3053 (1966); W. F. Brace, J. B. Walsh, W. T. Frangos, *ibid.* **73**, 2225 (1968).
4. W. F. Brace, *ibid.* **70**, 391 (1965).
5. W. R. Wawersik and W. F. Brace, *Felsmechanik Ingenieurgeol.* **3**, 61 (1971); H. Koide and K. Hoshino, *Jishin* **20**, 85 (1967); R. J. Willard and J. R. McWilliams, *Int. J. Rock Mech. Min. Sci.* **6**, 1 (1969); A. Timur, W. B. Hempkins, R. M. Weinbrandt, *J. Geophys. Res.* **76**, 4932 (1971); S. Baldridge and G. Simmons, *Eos* **52**, 342 (1971).
6. Grinding of a surface follows a procedure similar to that for preparation of a polished section. We used the following sizes of SiC or Al_2O_3 abrasive in the order shown: 130, 57, 30, 14, 8, and 1 μm.
7. After grinding, a specimen was placed in a Commonwealth Scientific ion milling instrument. An ionized argon beam (6 kv) bombarded the sample at an angle of 15° to 17°, knocking off individual atoms. The maximum intensity was 50 μa/mm^2. From 10 to 50 μm of material was removed from the ground surface by this bombardment. A somewhat uneven surface resulted, because thinning rates vary significantly from mineral to mineral. For example, quartz thinned approximately 10 times as fast as magnesian olivine. For granites, thinning times from 12 to 48 hours have produced satisfactory results. If thinning was continued too long, then relief was so great that detail was lost.
8. A JEOL model JSM-U3 scanning electron microscope was used to obtain magnifications of up to × 10,000. The secondary electron yield, which is sensitive to the topography of the surface and the voltage of the incident beam, was used to determine the image. The accelerating voltage was 25 kv.
9. W. F. Brace and A. S. Orange, *J. Geophys. Res.* **73**, 5407 (1968).
10. F. Birch, *ibid.* **66**, 2199 (1961); W. F. Brace and J. D. Byerlee, *Failure and Breakage of Rock* (American Institute of Mining, New York, 1967), p. 58; A. Nur and G. Simmons, *Int. J. Rock Mech. Min. Sci.* **7**, 307 (1970).

11. Supported by NSF grant GA-18342. D. Kohlstedt assisted us in sample preparation, and discussion of the results with T. R. Madden, J. B. Walsh, S. Baldridge, and P. Y. Robin was particularly helpful.

7 July 1972

Human Blood Monocytes: Stimulators of Granulocyte and Mononuclear Colony Formation in vitro

Abstract. *Human blood monocytes in a feeder layer or by use for conditioning medium produced a colony-stimulating factor capable of stimulating the in vitro growth of colonies of granulocytes and mononuclear cells from human and murine marrow. Lymphocytes and neutrophils did not stimulate colony formation, and medium conditioned by neutrophils was inhibitory. This suggests that the monocyte may control granulocyte proliferation and maturation.*

Colonies of granulocytes and mononuclear cells can be grown in vitro from the blood and marrow of animals (*1–3*) and man (*4–8*) in the presence of a colony-stimulating factor (CSF). Stimulation of colony growth can be achieved by various cell feeder layers (*1, 2, 4*), serums (*9, 10*), urine (*11*), and by conditioned medium prepared from various tissues (*12, 13*). Partial purification of CSF obtained from human urine (*14*) and mouse fibroblasts (*15*) reveals that it is a glycoprotein with a molecular weight of approximately 45,000.

Human blood leukocytes, either in a feeder layer (*4*) or by conditioning medium (*12*), will stimulate the growth of colonies from animal sources and at the present time are the best source of stimulation for growth of human colonies. However, it has not been determined which specific blood leukocyte is responsible for producing CSF. While it has been suggested that the neutrophil may be the source of CSF (*4*), this particular cell has also been reported to be inhibitory to colony growth (*16, 17*). The results of this study indicate that the blood monocyte is the cell responsible for CSF production and for the stimulation of granulocyte and mononuclear cell growth in vitro.

Eighty milliliters of blood, kept from coagulating with ethylenediaminetetraacetate was obtained by venipuncture from a healthy young adult and separated into a number of leukocyte fractions as follows.

Fraction 1: Mixed leukocytes and platelets. A sample of blood was mixed with one-fifth volume of 5 percent dextran-40 and allowed to sediment at room temperature for 1 hour. The supernatant, which was rich in leukocytes and platelets, was removed and washed three times with Seligmann's buffered salt solution (SBSS) (*18*).

Fraction 2: Mixed leukocytes without platelets. A sample of blood was defibrinated with glass beads for 30 minutes at room temperature and then handled in a manner identical to that used for fraction 1.

Fraction 3: Mononuclear cells and platelets. From another sample of blood, mononuclear cells were isolated by Ficoll-Hypaque density gradient separation (*19*). The mixture of mononuclear leukocytes and platelets was then washed three times with SBSS.

Fraction 4: Pure monocytes. A sample of cells from fraction 3 was passed through a sucrose gradient (*19*) in order to separate the mononuclear cells. The platelet-free mononuclear cells were then incubated in a concentration of 5×10^6 cells in 35-mm plastic tissue-culture dishes (Falcon Plastics) for 2 hours to allow the attachment of monocytes. Dishes were then washed vigorously with SBSS to remove nonadherent lymphocytes. Cells in one dish were stained with supravital stain to determine the leukocyte differential. The cell layer was then lysed with 1*N* NaOH and analyzed for DNA (*20*) to determine the number of cells in the monocyte monolayer.

Fraction 5: Lymphocytes. A sample of blood was incubated in 5 percent dextran containing iron particles to remove the phagocytic cells (*21*). The cells were then separated by the Ficoll-Hypaque gradient and processed as described above.

Fraction 6: Neutrophils. The neutrophils and red cells that passed through the Ficoll-Hypaque gradient in the preparation of fraction 3 were resuspended in SBSS containing 1 percent dextran. After sedimentation for 1 hour at room temperature the neutrophil-rich supernatant was washed three times with SBSS.

The differential count of each leuko-

Table 1. Ability of blood leukocytes to produce colony-stimulating factor.

Leukocyte fraction*	Differential leukocyte count†					Colonies‡
	Neutrophil	Lymphocyte	Monocyte	Eosinophil	Basophil	
1) Leukocytes with platelets	51.4	32.6	8.8	7.0	0.2	23 ± 2.8
2) Leukocytes	41.6	50.0	6.0	2.4		20 ± 4.0
3) Mononuclear cells	7.0	58.0	33.0		2.0	24 ± 3.2
4) Monocytes	2.0		98.0			35 ± 2.8
5) Lymphocytes		96.0	1.0		3.0	7.0 ± 1.2
6) Neutrophils	90.0	1.0		9.0		5.5 ± 2.4
None (control)						5.2 ± 1.6

* Fractions used to condition medium; 0.5×10^6 cells were incubated with each milliliter of medium for all fractions except 4, which contained 0.3×10^6 cells per milliliter. † Percentage of each cell type based on a differential of 500 cells. ‡ Number per 10^5 mouse marrow cells, mean ± standard error of three to five plates stimulated by 0.1 ml of conditioned medium.

cyte preparation is listed in Table 1. Each preparation contained more than 90 percent viable cells as determined by exclusion of nigrosine (*22*).

Feeder layers were prepared by the method described by Pike and Robinson (*4*). Cells from fractions 1, 2, 3, 5, and 6 were suspended in 0.5 percent agar and McCoy's culture medium, and 1-ml samples containing 10^6 cells per milliliter were plated in culture dishes 35×10 mm. Feeder layers from fraction 4 were prepared by directly overlaying the attached monocyte monolayer with 1 ml of agar.

Conditioned medium was prepared from fractions 1, 2, 3, 5, and 6 as described previously (*12*). From each cell fraction 10^6 cells were suspended per 2 ml of McCoy's 5A culture medium and incubated at 37°C in 7.5 percent CO_2. Conditioned medium from fraction 4 was prepared by overlaying the attached monocytes with 2 ml of McCoy's 5A medium. After 7 days of incubation, cells were removed by centrifugation and the supernatant (conditioned medium) was passed through a Millipore filter (pore size, 0.45 μm) and frozen at −20°C prior to testing for CSF.

The ability of specific leukocyte fractions and conditioned medium prepared from these fractions to stimulate in vitro colony growth was tested in the soft gel system for culturing mouse bone marrow described by Pluznik and Sachs (*1*) and by Bradley and Metcalf (*2*). Methylcellulose was used in place of agar (*12*). Human or murine marrow cells in a concentration of 10^5 per milliliter were suspended in 1.6 percent methylcellulose containing culture medium and 10 percent horse serum.

Colony-stimulating activity of specific cell feeder layers was determined by overlaying each feeder layer with 1 ml of the mixture of methylcellulose, serum, and cells. Conditioned medium from various leukocyte fractions was tested for CSF activity by adding 0.1 ml to each 0.9 ml of this mixture. One milliliter of the combined mixture was then plated in culture dishes 35×10 mm. All plates were incubated at 37°C in an atmosphere of 7.5 percent CO_2 for a period of 7 days for murine marrow and 16 days for human marrow. At that time the number of colonies (more than 50 cells) was counted with the aid of an inverted microscope. For morphological identification, individual colonies were removed with a Pasteur pipette, smeared between glass cover slips, and stained with Wright's stain.

Table 2. Stimulation of colony formation by feeder layers of blood leukocytes.

Composition of feeder layers	Colonies per 10^5 marrow cells	
	Human	Mouse
Mixed leukocytes	80 ± 4.2	33 ± 2.3
Monocytes	125 ± 5.5	62 ± 2.4
Lymphocytes	2.0 ± 0.6	6.0 ± 0.8
Neutrophils	0.0	0.4 ± 0.2
None (control)	0.0	8.0 ± 1.6

The separated leukocyte fractions and their ability to produce CSF and stimulate in vitro colony formation are given in Table 1. Nearly pure separation of monocytes (fraction 4), lymphocytes (fraction 5), and neutrophils (fraction 6) was achieved for preparing feeder layers and conditioning medium.

Colony-stimulating factor was produced only by those fractions containing monocytes. Conditioned medium prepared from pure monocytes stimulated (expressed as the mean ± standard error of the mean) 35 ± 2.8 colonies per 0.1 ml of medium, which was significantly greater ($P < .05$) than stimulation by the mixed leukocyte fraction. Fractions of lymphocytes (5) and neutrophils (6) did not produce CSF. In a separate experiment, five times as many neutrophils (2.5×10^6) were incubated per milliliter of medium. This medium likewise did not contain colony-stimulating activity. Medium conditioned by either 10^6 monocytes or 10^6 mixed leukocytes stimulated 47 ± 3.1 and 13 ± 2.3 colonies, respectively. Platelets did not appear to contribute to CSF production, since there was no significant difference in stimulating activity of CSF produced with or without platelets (fraction 1 versus fraction 2).

Stimulation of colony formation by feeder layers of separated leukocytes is given in Table 2. Colony formation was stimulated from human as well as murine marrow. As with conditioned medium, the greatest stimulation was by the pure monocyte fraction, which stimulated 120 ± 5.5 colonies from 10^5 human marrow cells and 62 ± 2.4 colonies from 10^5 murine marrow cells. This was significantly greater ($P < .02$) than stimulation by other cell fractions. Lymphocytes (fraction 5) and neutrophils (fraction 6) did not stimulate colony formation. Colonies stimulated by feeder layers of the monocyte fraction ranged between 50 to 1500 cells (mean, 600) for both the human and mouse colonies and were considerably larger than colonies stimulated by other cell fractions. Morphologically, colonies from mouse marrow were granulocytic and mononuclear. Colonies from human marrow consisted of either eosinophils, neutrophils, monocytes, macrophages, or a mixed cell type.

In order to determine whether conditioned medium prepared from neutrophils might be inhibitory, 0.1 ml of this medium was added to culture plates being stimulated with conditoned medium from mouse fibroblasts (*15*). Conditioned medium prepared by incubating 0.5×10^6 neutrophils per milliliter of medium resulted in a slight but statistically insignificant decrease in colony formation. However, the addition of 0.1 ml of conditioned medium prepared from 2.5×10^6 neutrophils per milliliter of medium significantly inhibited colony formation and resulted in a decrease from 115 colonies in control plates to 85 colonies in plates containing conditioned medium from neutrophils ($P < .05$).

The results of this study indicate that the monocyte is the blood leukocyte responsible for the production of a CSF that is capable of stimulating the in vitro growth of granulocytes and mononuclear cell colonies from blood and marrow of animals and man. Colony-stimulating factor was produced only by those cell fractions containing monocytes, with the greatest activity being obtained from the "pure" monocyte fraction. This was true whether feeder layers of specific leukocyte fractions or conditioned medium prepared from these fractions was used to stimulate colony growth. Lymphocytes and neutrophils did not stimulate colony growth either in a feeder layer or by use for conditioning medium. Platelets did not appear to contribute to CSF production since no significant difference in activity was noted when colonies were stimulated with or without platelets.

Neutrophils not only failed to produce CSF but medium incubated with 2.5×10^6 cells per milliliter was inhibitory to colony growth. This finding is similar to previous observations (*16, 17*) that neutrophils may be inhibitory to colony formation.

Colony-stimulating factor has been obtained from a number of other tissues, including mouse embryo, lung, uterus, muscle, and liver (*13*). The finding that the blood monocyte is the leukocyte responsible for CSF production may help explain its presence in these other tissues. It is conceivable

that when monocytes leave the blood and become tissue macrophages, they retain the ability to generate and release CSF. The wide distribution of these cells throughout the body would fit with the finding of CSF in multiple organs. Variation in activity in different organs may reflect the concentration of monocytes and macrophages in these tissues.

The finding that the monocyte stimulates the in vitro production of granulocytes is of considerable interest. However, this finding might not be totally unexpected since there are a number of physiological and biochemical events (*23*) as well as the growth pattern of these cells in vitro (*24*) which indicate a close relation between these cell lines. This finding may also be true in vivo since it is known that recovery from marrow aplasia or hypoplasia is nearly always preceded by the appearance of monocytes followed by the return of granulocytes. It may well be that the monocyte-macrophage system which is capable of performing many specialized functions (*25*) is also involved with the control of granulocyte proliferation and maturation. However, as yet, there is no convincing evidence that CSF is a physiological regulator of in vivo cell production.

PAUL A. CHERVENICK
ALBERT F. LOBUGLIO
Department of Medicine, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15213, and *Department of Medicine, Ohio State School of Medicine, Columbus 43210*

References and Notes

1. D. H. Pluznik and L. Sachs, *J. Cell. Comp. Physiol.* **66**, 391 (1965).
2. T. R. Bradley and D. Metcalf, *Aust. J. Exp. Biol. Med.* **44**, 287 (1966).
3. J. C. Marsh, M. Levitt, A. Katzenstein, *J. Lab. Clin. Med.* **79**, 1041 (1972).
4. B. L. Pike and W. A. Robinson, *J. Cell. Physiol.* **76**, 77 (1970).
5. P. A. Chervenick and D. R. Boggs, *Blood* **37**, 131 (1971).
6. K. B. McCredie, E. M. Hersh, E. J. Freireich, *Science* **171**, 293 (1971).
7. N. N. Iscove, J. S. Senn, J. E. Till, E. A. McCulloch, *Blood* **37**, 1 (1971).
8. P. A. Chervenick, L. D. Ellis, S. F. Pan, A. L. Lawson, *Science* **174**, 1134 (1971).
9. R. Foster, Jr., D. Metcalf, W. A. Robinson, T. R. Bradley, *Brit. J. Haematol.* **15**, 147 (1968).
10. P. A. Chervenick, *J. Lab. Clin. Med.* **79**, 1014 (1972).
11. W. A. Robinson, E. R. Stanley, D. Metcalf, *Blood* **33**, 396 (1969).
12. P. A. Chervenick and D. R. Boggs, *Science* **169**, 691 (1970).
13. T. R. Bradley, E. R. Stanley, M. A. Sumner, *Aust. J. Exp. Biol. Med. Sci.* **49**, 595 (1971).
14. E. R. Stanley and D. Metcalf, *Proc. Soc. Exp. Biol. Med.* **137**, 1029 (1971).
15. P. E. Austin, E. A. McCulloch, J. E. Till, *J. Cell. Physiol.* **77**, 121 (1971).
16. J. S. Haskill, R. D. McKnight, P. R. Galbraith, *Blood* **38**, 788 (1971).
17. M. Paran, Y. Ichikawa, L. Sachs, *Proc. Nat. Acad. Sci. U.S.A.* **62**, 81 (1969).
18. P. B. Noble, J. H. Cutts, K. K. Carroll, *Blood* **31**, 66 (1968).
19. R. J. Perper, T. W. Zee, M. M. Mickelson, *J. Lab. Clin. Med.* **72**, 842 (1968).
20. G. Ceriotti, *J. Biol. Chem.* **198**, 297 (1952).
21. S. Thierfelder, *Vox Sang.* **9**, 447 (1964).
22. J. P. Kaltenbach, M. H. Kaltenbach, W. B. Lyons, *Exp. Cell Res.* **15**, 112 (1958).
23. L. O. Leder, *Blut* **16**, 86 (1967).
24. D. Metcalf, *J. Cell. Physiol.* **77**, 277 (1971).
25. N. N. Pearsall and R. S. Weiser, *The Macrophage* (Lea & Febiger, Philadelphia, 1970), pp. 71–92.
26. Supported in part by grants from the American Cancer Society (ET-10B), Health Research and Services (N-66), PHS (AM 14352-03), and National Cancer Institute (1-R01-CA 13381-01). P.A.C. is a Leukemia Society of America Scholar. We thank J. Allalunis, M. Welt, T. Scammon, and H. Gordecki for their excellent assistance.

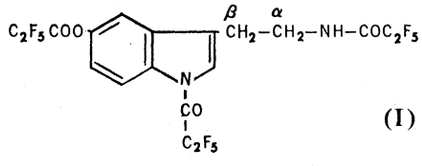
10 July 1972

Gas Chromatographic–Mass Spectrometric Assay of Four Indole Alkylamines of Rat Pineal

Abstract. *Gas chromatography–mass spectrometry was used to quantitate serotonin, N-acetylserotonin, 5-methoxytryptamine, and melatonin in single rat pineal glands. After gas chromatographic separation, the ion density of specific fragments of each indole was measured with mass spectrometry. Sensitivity of this indole assay is of the order of 10^{-12} to 10^{-13} mole. Routinely, specificity is based on gas chromatographic retention time and the recording of the ion density generated by specific fragments. Absolute identification of the extracted indoles was based on multiple ion detection.*

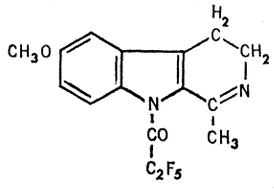
The technique of quantitative gas chromatography–mass spectrometry (*1*) is applied to measure the following indole alkylamines: serotonin, *N*-acetylserotonin (NAS), 5-methoxytryptamine (5MT), and melatonin. Serotonin, a putative neurotransmitter, is ubiquitous in the central nervous system, and existing methods are not absolutely specific (*2*). Until recently (*3*), it was believed that NAS, 5MT, and melatonin were uniquely located in the pineal gland. The specificity of the methods used is questionable since they involve either bioassay in the case of melatonin (*4*) or elaborate organic solvent extraction and reaction with *o*-phthalaldehyde to form fluorophores with the same emission and activation spectra (*5*).

To form indoles with the appropriate vapor pressure for gas chromatography, they are reacted with pentafluoropropionic anhydride (PFPA) to obtain acylation of hydroxyl and primary and secondary amine groups. When serotonin reacts with PFPA, the product (I) contains three pentafluoropropionyl (PFP) groups (molecular weight, 614). The product obtained with 5MT contains two PFP groups (molecular weight, 482),



(I)

and the internal standard α-methylserotonin (α-MS) contains three PFP groups (molecular weight, 628) (*6*). Those indoles with an *N*-acetyl side chain react with PFPA, forming a compound structurally similar to a β-carboline. The reaction product (II) of melatonin contains one PFP group (molecular weight, 360).



(II)

N-Acetylserotonin adds on two PFP groups (molecular weight, 492) and the internal standard *N*-acetyltryptamine (NAT), one PFP group (molecular weight, 330).

The structures and fragmentation patterns of these acylated derivatives have been determined with a LKB 9000 gas chromatograph–mass spectrometer. Gas chromatograph conditions were: 9-foot (1 foot = 0.3 m) glass column (inside diameter, 2 mm) packed with OV-17, 3 percent on Gas Chrom Q, 100 to 120 mesh; flash heater, 290°C; oven, 210°C; and helium flow, 15 ml/min. All the compounds have similar gas chromatographic properties and are completely resolved from each other (Table 1). Mass spectrometry conditions were: molecular separator, 250°C; ion source, 290°C; electron energy, 80 ev; trap current, 60 μa; and electron multiplier, 3.7 kv.

Mass spectral analysis of the indole-PFP derivatives revealed that their fragmentation pattern depends on the presence or absence of the *N*-acetyl group. Indoles with an *N*-acetyl group