Neutrophil Products That Inhibit Cell Proliferation: Relation to Granulocytic “Chalone”

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Various investigators have proposed the existence of a “chalone” or product of mature granulocytes which inhibits the replication of granulocytic progenitors in a tissue-specific, species-nonspecific regulatory system. We tested this system utilizing purified populations of human leukocytes and a variety of proliferating target cell populations. Granulocyte-conditioned medium potently inhibited $^3$H-thymidine incorporation in normal mouse bone marrow, in mouse erythroid precursors from spleens of phenylhydrazine-treated animals, and in human leukemic myeloblasts. This effect occurred without demonstrable cytotoxicity. Human marrow was only slightly inhibited and mitogen-stimulated proliferating lymphocytes were unaffected. Granulocyte-conditioned medium was also inhibitory in colony-forming assays for erythroid precursors but not for granulocyte precursors. It also reduced the mitotic index of proliferating erythroid progenitors in mouse spleen. Inhibitory activity was separable into at least two components on the basis of stability during heating and ultrafiltration, and in susceptibility to digestion by proteolytic enzymes. On the basis of these observations, we concluded that human granulocytes contain potent inhibitors of growth and $^3$H-thymidine uptake of some proliferating hematopoietic cells, but these are not true granulocyte “chalones.”

In 1960, Bullough and Lawrence provided experimental evidence for the control of epidermal cell mitosis by products of mature cells in culture in vitro. They evolved the concept of a tissue-specific product of differentiated cells that served as a mitotic inhibitor of early cells of the same line. They termed the inhibitor a “chalone” (from the Greek, “to loosen” or “make slack”). Since then, a number of experimenters have suggested the existence of chalones specific for various tissues. More recently, various investigators have emphasized theoretical reasons why granulopoiesis should be under negative feedback control and have adduced evidence for the existence of granulocyte chalones. However, the diversity of model systems, methodologic limitations, and frequently inadequate experimental design have made interpretations difficult.

Rytömaa and Kiviniemi, Paukovitz, and Bateman have presented evidence suggesting that rat and calf serum solutions “conditioned” by granulocyte-rich cell suspensions and spleen homogenates inhibit DNA synthesis in developing myeloid precursors in short-term cultures of rat bone marrow cells. This “chalone” material has been partially purified by gel filtration, ultrafiltration, and electrophoresis. It is reported to be a polypeptide of a molecular weight of approximately 4000–5000 daltons, which is unstable at 4°C, with
activity directed against all recognizable proliferative granulocyte progenitors. Inhibition of myeloid precursors is said to be transient, readily reversible, and not associated with cytotoxicity.8

Both Metcalf and Moore9 and Lajtha10 expressed doubt regarding the chalonal nature of these substances derived from leukocyte-conditioned medium. For example, the inhibition of DNA synthesis observed in Rytömaa’s studies was greater than the 20%–30% that could be accounted for on the basis of selective inhibition of myeloid precursors. In similar studies, Lajtha found inhibition of 50% or 60%.

Data from diffusion chamber studies in vivo relating to granulocyte chalones are also difficult to interpret.11-13 Furthermore, pure cell populations have not been used as a source of inhibitory activity in these studies. Additionally, lysosomal extracts from leukocytes may inhibit the growth of cultured cell lines.14 These considerations suggest caution in the interpretation of the data purporting to demonstrate specific granulocyte chalones.

A variety of inhibitors of granulopoiesis have been reported to occur in mammalian serum. Normal mouse15,16 and human17-19 sera contain lipoprotein inhibitors of mouse myeloid stem cells (CFU-C). Inhibitory activity is precipitated during dialysis and is destroyed by heat treatment.16 This serum inhibitor, which is similar to that found in human urine, may block the action of colony-stimulating activity (CSA). No definitive evidence exists that these diverse “inhibitors” found in serum and urine are physically or physiologically equivalent to a granulocyte chalone. Furthermore, the multiplicity of reports of fluctuation of serum and urine inhibitor levels with clinical disease and therapy15,20-22 have contributed little to providing a firm basis for the granulocyte chalone concept. Nevertheless, the concept of negative feedback regulation of hematopoiesis is important and deserves careful scrutiny.

For this reason, we undertook detailed studies to examine the effects of products of mature granulocytes on hematopoietic cell proliferation. We observed definite inhibitory activity against erythroid precursors but no unequivocal activity against normal neutrophil progenitors. 3H-Thymidine incorporation into three acute leukemic cell populations was also inhibited. We have tentatively termed the factors involved neutrophil-derived inhibitory factors (NDIF). At least two separable inhibitory activities were observed, neither of which had the characteristic of a purported granulocyte chalone.

**MATERIALS AND METHODS**

**Preparation of Neutrophil-derived Inhibitory Factors (NDIF)**

Cells from 500 ml of heparinized venous blood from 14 normal adults were separated into neutrophil (PMN), mononuclear leukocyte (MNL), and erythrocyte (RBC) fractions by sequential density gradient centrifugation on Ficoll-Hypaque23 and Dextran sedimentation. Cells were thrice washed by centrifugation at 150 g for 10 min in physiologic saline. RBC were removed from the PMN fraction by hypotonic lysis.24 Criteria of purity of each fraction were defined by light microscopy of Giemsa-stained preparations and histochemistry with chloroacetate and α-naphthyl butyrate stains.25 The MNL population was further separated into adherent (predominantly monocyte) and nonadherent (predominantly lymphocyte) fractions by incubation on plastic Petri dishes at 37°C for 2 hr. The PMN population was 91% ± 4% (mean ± SD) pure (n = 7) with 7% ± 2% eosinophils and less than 1% MNL and RBC. The nonadherent MNL population contained 89% ± 8% lymphocytes, 2% ± 2% PMN, and 9% ± 2%.
monocytes. The adherent MNL population contained 70±7% monocytes, 21±4% lymphocytes, and 4±3% PMN. The RBC fraction was 100% pure. Platelets were not a significant contaminant.

Purified cell populations were usually incubated at 37°C in sterile 0.9% saline with penicillin and streptomycin at a concentration of 50 x 10⁶ cells/ml for 16 hr. Variations in the incubation conditions included cell concentrations of 1 to 100 x 10⁶/ml, incubation times of 2-36 hr, and the exclusion of antibiotics. After incubation, the cells were removed by centrifugation at 750 g. After testing for microbial contamination, the cell-free supernatant was stored at -20° or -70°C in small aliquots until assayed for NDIF activity.

**Assay of NDIF Activity**

**³H-Thymidine (³H-TdR) incorporation.** In the standard assay, 4 x 10⁵ bone marrow cells from male 20-30-g Swiss-Webster mice were incubated in triplicate or quadruplicate in a volume of 0.44 ml of 10% aged fetal calf serum (FCS) in normal saline containing 1-100 μl of NDIF and 0.4 μCi ³H-TdR (6.7 Ci/mM, 0.35 μg/ml). After 2 hr of incubation at 37°C, isotope incorporation was stopped with iced balanced salt solution. Some cells were prepared for autoradiography, and others were pelleted and resuspended in 7.5% cold trichloroacetic acid (TCA) and collected and washed with TCA and ethanol on Millipore filters (No. AP2502500). Tritium incorporation was determined in a Beckman liquid scintillation spectrometer. Results were expressed as the mean ± SD of the percent inhibition relative to control assays containing saline or RBC-conditioned medium in lieu of NDIF. FCS aged for 1 wk at room temperature was used in preference to fresh-frozen FCS because of inhibitory activity in the latter.⁷

Other target cell populations used for the standard ³H-TdR NDIF assay included: (1) marrow cells enriched in myeloid precursors from mice that had been hypertransfused with RBC over a period of 9 days to a hematocrit of 58%-67% (mean 64%); (2) spleen cell suspensions enriched in erythroid precursors from mice that had received phenylhydrazine, 1 mg intraperitoneally every other day for 3 of the 5 previous days;²⁶ (3) blast cells of >96% purity from three patients with acute myelocytic leukemia (AML); (4) suspensions of normal human bone marrow prepared as previously described;²⁷ and (5) human lymphocytes 1 x 10⁶/ml incubated with an optimal concentration of phytohemagglutinin-P (PHA, Difco) for 72 hr prior to assay.²⁸ All cell numbers except lymphocytes were adjusted to 4 x 10⁵ per assay. Cells were prepared for autoradiography as previously described²⁷ using Kodak NTB emulsion and development times of 7-9 days. Areas free of cells were used to establish background grain counts, which were negligible.

**Clonogenic assays.** NDIF or saline in volumes of 0.10-100 μl was added to agar or methylcellulose assay cultures of mouse myeloid stem cells (monocyte-granulocyte progenitors, CFU-C) and erythroid progenitors (CFU-E). CFU-C and CFU-E assays were performed as previously described.²⁹ ³¹

**Mitotic index.** Cells freshly isolated from normal mouse bone marrow or phenylhydrazine-treated mouse spleens were incubated in complete tissue culture medium with colcemid 0.1 μg/ml in the presence or absence of 1/10 volume NDIF. At intervals of up to 7 hr, aliquots were removed and stained with Giemsa, and 5000 cells were counted to determine the mitotic index (mitoses/100 cells x 100).

**Manipulation of NDIF.** Before assay, various NDIF preparations were subjected to the following procedures: freezing and thawing three times; boiling for 5 min; filtration through Amicon filters at exclusion pore sizes of 2000 and 10000 daltons; dialysis through Spectrapore membranes, exclusion pore size 3500 daltons; and ultracentrifugation at 70,000 g for 3-4 hr. Other preparations of NDIF were incubated with trypsin, pepsin, or papain for 2 or 18 hr as described by others.³¹

**RESULTS**

**Sources of NDIF**

Partially purified (91% ± 4%) populations of human PMN were aseptically incubated in saline and the cell-free medium was assayed for NDIF activity expressed as the percentage inhibition of ³H-TdR incorporation by mouse bone...
marrow cells. Inhibitory activity was linearly related to PMN concentrations between 2 and 50 × 10⁶ cells/ml (six experiments, Fig. 1A) and gradually increased from 20% to 45% between 2 and 36 hr of incubation (Fig. 1B). Lysates of PMN prepared by freezing and thawing or sonication prior to incubation (0 time) had activity comparable to that observed in the supernatant of 24-hr incubations. An incubation time of 16 hr at a cell concentration of 50 × 10⁶/ml was chosen as a standard.

When tested at comparable cell concentrations (50 × 10⁶/ml) in four experiments, medium conditioned by RBC had no detectable inhibitory activity, and medium from lymphocyte-enriched MNL populations had 0–21% of the PMN activity. Monocyte-enriched MNL supernatants had 22%–50% of the activity observed in PMN-conditioned medium. The contribution of contaminating PMN (a mean of 2%–4%) to this inhibitory activity could not be excluded. Subsequently, only PMN-conditioned medium was examined.

Characteristics of the NDIF-³H-TdR Assay

When the effect of various concentrations of NDIF was assayed for inhibition of ³H-TdR incorporation in normal mouse bone marrow, a parabolic curve was observed (10 experiments, Fig. 2A) which appeared as a straight line on a semilogarithmic plot (Fig. 2B). In three experiments, activity was reliably detected in as little as 1 µl or the equivalent of medium conditioned by 5 × 10⁶ PMN. The protein content of NDIF in these experiments was 1.7 µg/ml.

Inhibition of ³H-TdR incorporation into cells was independent of incubation time of the NDIF with target marrow between 2 and 6 hr (Fig. 3A); 2 hr was therefore chosen for convenience. The NDIF itself had been prepared by incubating PMN for 16 hr prior to harvest.

The percentage inhibition of ³H-TdR incorporation decreased slightly with increasing target cell concentrations between 8 × 10⁶ and 20 × 10⁶ cells per 0.44-ml assay (Fig. 3B). No difference was observed between target cell concentrations of 8 × 10⁶ and 4 × 10⁶ cells, and 4 × 10⁶ was chosen as a standard.
Fig. 2. (A) Various volumes of PMN-conditioned medium (NDIF) were incubated with 4 x 10^5 mouse bone marrow cells and the inhibition of incorporation of ^3H-TdR (mean ± SD) was recorded for 10 experiments. (B) NDIF was assayed as in A, except that aliquots were tested after boiling for 5 min (heat stable). The heat-labile curve was derived from subtraction of heat-stable from total activity. NDIF volume is plotted on a logarithmic scale.

The percentage inhibition of ^3H-TdR incorporation was also decreased slightly as the total amount of isotope added was increased (Fig. 3B). ^3H-TdR incorporation was greater than 90% inhibitible by nonradioactive (cold) thymidine at 1.2 μg/ml. This amount of cold thymidine could have contaminated NDIF preparations without being detectable spectrophotometrically.

**Target Cells Inhibited by NDIF**

One of the putative characteristics of a chalone is its tissue specificity and its species nonspecificity. Therefore, in looking for a potential granulocyte chalone, we examined a variety of proliferating hematopoietic tissues that varied widely in their content of granulocyte (myeloid) precursors. Mouse marrow was enriched in myeloid precursors by erythropoietic suppression from hypertransfusion. Mouse spleen cells were enriched in erythroid precursors by

Fig. 3. (A) Effect of various incubation times of target cells with NDIF on the inhibition of incorporation of ^3H-TdR. (B) Effect of various concentrations of ^3H-TdR and various concentrations of mouse marrow target cells on the inhibition of incorporation of ^3H-TdR.
prior treatment with phenylhydrazine. Other target populations examined were normal human marrow cells, human leukemic myeloblasts, and human lymphocytes undergoing mitogen-stimulated blastogenesis. The results are shown in Table 1 and Fig. 4.

At all concentrations of NDIF between 1 and 100 μl per 0.44-ml assay, inhibition of 3H-TdR incorporation into hypertransfused mouse marrow enriched in myeloid precursors was less than in the normal heterogeneous mouse bone marrow. In addition, inhibition by NDIF was as great in erythropoietically enriched, phenylhydrazine-treated spleen cells as in normal mouse bone marrow. These observations suggest that proliferating erythroid precursors are susceptible to the inhibitory action of NDIF.

Incorporation of 3H-TdR into normal human marrow was inhibited only at higher concentrations. In three experiments, NDIF stimulated 3H-TdR incor-

### Table 1. NDIF Inhibition of 3H-TdR in Various Target Cells

<table>
<thead>
<tr>
<th>Target Cells</th>
<th>Purity (%)</th>
<th>Inhibition of 3H-TdR (%) at Various NDIF Concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 μl</td>
</tr>
<tr>
<td>Normal mouse bone marrow</td>
<td>54 ± 6 myeloid*</td>
<td>7 ± 6(3)</td>
</tr>
<tr>
<td>Hypertransfused mouse marrow</td>
<td>81 ± 5 myeloid†</td>
<td>2 ± 2(3)</td>
</tr>
<tr>
<td>Phenylhydrazine-treated spleen</td>
<td>10 ± 5 myeloid</td>
<td>17 ± 13(3)</td>
</tr>
<tr>
<td>Human bone marrow spleen</td>
<td>62 ± 6 erythroid</td>
<td>0(3)</td>
</tr>
<tr>
<td>Leukemic myeloblasts</td>
<td>&gt; 96</td>
<td>14 ± 18(3)</td>
</tr>
<tr>
<td>Human lymphocytes (PHA-stimulated)</td>
<td>95 lymphoid</td>
<td>&lt; 1(3)</td>
</tr>
</tbody>
</table>

*Myeloid: all identifiable granulocytic cells.
†Erythroid: all identifiable nucleated red cell precursors.
§Slight stimulation; see Fig. 4.

![Fig. 4. Effect of various amounts of NDIF on inhibition of 3H-TdR incorporation into various target cells.](image-url)
Table 2. Comparison of NDIF Effect on \(^{3}\)H-TdR Incorporation Determined by Scintillation Counting and Autoradiography

<table>
<thead>
<tr>
<th>Target Marrow</th>
<th>Addition</th>
<th>Labeling Index (%)</th>
<th>Inhibition of (^{3})H-TdR dpm (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal human 1</td>
<td>Saline</td>
<td>5.0 ± 1.2</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>NDIF</td>
<td>3.8 ± 1.2</td>
<td>35 ± 3</td>
</tr>
<tr>
<td>Normal human 2</td>
<td>Saline</td>
<td>5.3 ± 1.2</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>NDIF</td>
<td>8.3 ± 2.1</td>
<td>38 ± 3</td>
</tr>
<tr>
<td>Normal mouse</td>
<td>Saline</td>
<td>15.6 ± 2.2</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>NDIF</td>
<td>16.7 ± 2.3</td>
<td>20 ± 2</td>
</tr>
</tbody>
</table>

In experiments using two different human bone marrows or pooled mouse bone marrow, the effects of NDIF on incorporation of \(^{3}\)H-TdR radioactivity determined by autoradiography and by scintillation counting were compared. The results, shown in Table 2, demonstrated little difference in autoradiographic labeling indices when simultaneous total incorporation of radioactivity was substantially reduced. In the autoradiography experiments, no attempt was made to discriminate between labeled myeloid and erythroid precursors because of the unreliability of discriminating the early morphologic forms of these series.

**Effect of NDIF in Other Cell Proliferation Systems**

In assessing the biologic activity of NDIF preparations, it seemed important to use assay systems independent of the incorporation of \(^{3}\)H-TdR because of the susceptibility of the latter system to distortion by cold thymidine, changes in nucleotide pool size, changes in transport, and other phenomena. Available assays of CFU-C and CFU-E are reliable and seemed appropriate for determining the effects of NDIF on proliferating hematopoietic cells.

It was first determined that neither of these assays was inhibited by cold thymidine in the range of 0.03–0.3 \(\mu\)g/ml. The results of testing various NDIF preparations and concentrations in the CFU-E assay are shown in Table 3 (six experiments) and Fig. 5 (a single dose–response curve). A volume of 0.1 \(\mu\)l produced a clear inhibition of CFU-E. This volume was the equivalent of material from 5000 PMN or, if the active factor was a protein, equivalent to 80 ng/ml. Below this volume inhibitory effect was equivocal. Colony morphology in the presence of NDIF in the CFU-E assays was no different from controls. Only the data for stimulation with 0.5 U/ml erythropoietin are shown in Fig. 5 and
Table 3. Effect of NDIF on Mouse Bone Marrow CFU-E

<table>
<thead>
<tr>
<th>Volume of NDIF (µl)</th>
<th>Inhibition of CFU-E (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>22.2 ± 7.6</td>
<td>4</td>
</tr>
<tr>
<td>1</td>
<td>25.8 ± 12.4</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>57.2 ± 9.2</td>
<td>5</td>
</tr>
<tr>
<td>100</td>
<td>81</td>
<td>2</td>
</tr>
</tbody>
</table>

*All cultures contained 0.5 U/ml sheep plasma erythropoietin. In the absence of NDIF, control cultures formed 187–272 (mean 230) erythroid colonies per 5 x 10⁴ mouse marrow cells.

Table 3. Similar observations were made at 0.1 U/ml of erythropoietin. In contrast, in four experiments NDIF in volumes up to 100 µl had no demonstrable inhibitory effect on mouse marrow myeloid colony formation (CFU-C), and in four other experiments it had no effect on human CFU-C (data not shown).

**Mitotic Index**

Normal mouse bone marrow was incubated with colcemide in the presence or absence of NDIF for up to 7 hr. No effect of NDIF on the mitotic index was noted (Table 4). In these studies no attempt was made to differentiate the line of origin of the cells containing mitoses, since morphologic identification was unreliable in the absence of intact nuclear morphology.

In a subsequent series of experiments NDIF produced a clear reduction of mitotic index of phenylhydrazine-treated mouse spleen cells after 6 hr of incubation (Table 4; p < 0.01). In these studies more than 90% of the cells were erythroid.

**Properties of NDIF**

Preparations of NDIF retained activity in the ³H-TdR assay of normal mouse marrow when stored at −20°C for at least 6 wk. When boiled, considerable NDIF activity was lost. Little heat-stable activity was observed when small volumes of NDIF were used in the thymidine assay (Fig. 2B). At volumes greater than 25 µl, more than half the NDIF activity was heat stable. With marrow cells from normal man as the target, essentially all inhibitory activity was heat stable. Human leukemic myeloblasts and phenylhydrazine-treated mouse spleen cell suspensions resembled normal mouse marrow in regard to heat-

![Fig. 5. Various volumes of NDIF were tested for inhibition of erythroid colony (CFU-E) growth from a single mouse bone marrow in methylcellulose in the presence of maximally stimulating concentrations of erythropoietin.]
Table 4. Effect of NDIF on Mitotic Index of Mouse Hematopoietic Cells in Vitro

<table>
<thead>
<tr>
<th>Cells</th>
<th>Duration of Incubation (hr)</th>
<th>Mitotic Index (%)</th>
<th>Control</th>
<th>NDIF</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal mouse bone marrow</td>
<td>2</td>
<td>1.8 ± 0.3</td>
<td>2.1 ± 0.3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>4.0 ± 0.2</td>
<td>3.7 ± 0.3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>4.6 ± 0.2</td>
<td>5.4 ± 0.4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>4.4 ± 0.3</td>
<td>4.4 ± 0.3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Phenylhydrazine mouse spleen</td>
<td>2</td>
<td>9.3 ± 1.4</td>
<td>12.4 ± 2.5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>16.0 ± 1.5</td>
<td>14.5 ± 3.5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>18.7 ± 1.3</td>
<td>11.3 ± 1.5</td>
<td>4</td>
<td></td>
</tr>
</tbody>
</table>

stable and heat-labile inhibitory activities of NDIF, with prominent heat-labile activity below 50 μl.

In the CFU-E assay 30%–60% of inhibitory activity was lost by boiling NDIF preparations.

Two NDIF preparations were centrifuged at 70,000 g for 3–4 hr in five different experiments. An average of 80% of the total activity assayed by 3H-TdR in normal mouse marrow was recoverable in the supernatant.

In two experiments, NDIF preparations were dialyzed in Spectrapore membranes (3500 daltons exclusion) against saline. Activity was found equally distributed across the membrane. A total of 10 experiments were performed with a high-pressure Amicon ultrafiltration apparatus. A mean of 55% of the original inhibitory activity was found in a less than 2000 molecular weight filtrate, and 76% in a less than 10,000 molecular weight filtrate. Activity in the filtrate was entirely heat stable when assayed by the 3H-TdR mouse assay. Heat-labile activity was not consistently recoverable. When filtered through 0.2-μm Millipore filters, all heat-labile activity was lost and all heat-stable activity recovered.

In three experiments, NDIF was incubated with pepsin, trypsin, papain, or pronase for 2 or 18 hr, and activity was subsequently determined with the 3H-TdR mouse marrow assay. All appropriate controls for the effects of incubation with buffers and for the effects of enzyme on the assay system were included. The heat-stable inhibitory activity was unaffected by digestion. The effect of other proteolytic enzymes on heat-labile activity was not investigated.

The ether extraction method of Chan and Metcalf used in removing granulopoietic inhibitors from serum was tested on NDIF preparations. Inhibitory activity was unaffected by such extraction (three experiments, data not shown).

DISCUSSION

Saline extracts of relatively pure populations of human granulocytes have been demonstrated to contain one or more factors that inhibit thymidine incorporation into a variety of hematopoietic target tissues in vitro. These factors have been tentatively designated neutrophil-derived inhibitory factors (NDIF). The sensitivity of various target tissues ranked in descending order was (1) human leukemic cells, (2) normal mouse bone marrow and phenylhydrazine-pretreated mouse spleen, (3) hypertransfused mouse bone marrow,
and (4) normal human bone marrow. Little activity was observed against PHA-stimulated lymphocytes. NDIF also potently inhibited mouse erythroid colony growth in cultures of mouse bone marrow but had little inhibitory effect on the development of CFU-C. Inhibition of $^3$H-TdR incorporation occurred without significant cytotoxicity in short (2-hr) or intermediate (20-hr) incubations. NDIF had no detectable effect on either mitotic index after 7 hr of incubation or on autoradiographically determined $^3$H-TdR labeling index of normal mouse bone marrow. In contrast, NDIF reduced the mitotic index of erythropoietic cells of mouse spleen incubated in vitro for 6 hr.

The impressive difference between total $^3$H-TdR incorporation and autoradiographically determined labeling indices in NDIF-treated hematopoietic cells (Table 2) is consistent with prolongation of S phase but does not prove that this is the site of activity of NDIF.

NDIF activity was partially destroyed by boiling. Heat-stable NDIF was dialyzable through a 3500-dalton exclusion membrane, and resisted digestion with papain, pepsin, trypsin, and pronase. Heat-labile NDIF was destroyed by pronase and was lost on Millipore filtration.

Assays of chalone-like materials based on inhibition of $^3$H-TdR incorporation are susceptible to several artifacts: dilution of isotope with cold thymidine, changes in nucleotide pool size, interference with phosphorylation and transport, and simply cell death.32

Several of the properties of NDIF cannot be readily explained solely by thymidine contamination: its target organ specificity in $^3$H-TdR incorporation studies, its effects on mitotic activity, its inhibitory effects in cell growth assays, and its partial heat lability. For the same reasons, an effect on thymidine kinase activity in extracts is probably of little consequence. Moreover, this enzyme would have been destroyed by boiling and protease digestion. Furthermore, in five preliminary experiments the incorporation of $^3$H-deoxyuridine was suppressed even more than that of $^3$H-TdR.

By spectrophotometric analysis, the maximum possible thymidine concentration in the extract was $10^{-5} M$. This concentration of thymidine reduced $^3$H-TdR uptake > 90% when added in vitro to the isotope incorporation assay of normal mouse marrow and human lymphocytes. However, concentrations of cold thymidine 2–10 times this amount failed to inhibit erythroid colony formation in methylcellulose.

Several lines of evidence also suggest that NDIF does not kill cells. After 2-hr and 20-hr incubations of target cells with extract, viability by trypan blue exclusion was no different from controls. The biologic specificity of its effects also argues against general cytotoxicity, with greater effect on erythroid precursors and leukemic myeloblasts than granulocyte progenitors. Lymphocytes were not substantially affected in their rate of incorporation of $^3$H-TdR below 50 $\mu$l of extract, and human marrow cells were stimulated in this concentration range.

In view of these findings, we conclude that NDIF probably contains several activities, at least some of which are potent inhibitors of proliferation of susceptible cells.

The biochemical characterization of NDIF is incomplete. However, it appears to contain at least two physically distinct substances separable by ultra-
centrifugation. The material in the < 10,000-dalton fraction that is heat stable and dialyzable generally conforms to the specifications described by Rytömaa and Kiviniemi⁷⁻⁸ and Paukovitz.⁵⁻⁶ They maintain, on the basis of chromatographic methods, that granulocyte chalone is a small protein or polypeptide. Our finding of protease resistance would appear to be in conflict with their observations. However, they did not subject their chalone to protease digestion, and it is known that some small polypeptides may be resistant to proteases because of unusual amino acid sequences.

The heat-labile inhibitor could possibly be the lipoprotein inhibitor found by Chan et al.¹⁶⁻¹⁸ in mouse and human serum, but preliminary experiments with ether extraction suggest that this is unlikely. It is curious that Rytömaa and Kiviniemi, and Paukovitz found only the low molecular weight inhibitor in the conditioned medium of various granulocyte preparations. It is possible that a high molecular weight, heat-labile inhibitor was inadvertently lost by filtration for sterilization, by adherence to dialysis membrane, or by chromatographic procedures. Rytömaa and Kiviniemi filtered their material. Additionally, these investigators failed to utilize purified granulocyte preparations as starting material.

The characteristics of a chalone as described by Bullock are as follows:¹ (1) mitotic inhibition by negative feedback, (2) organ-specific origin, (3) target organ specificity, and (4) species nonspecificity. The granulocytic chalone must be distinguished from numerous other inhibitory substances from serum, granulocytes, and spleen that appear to be a heterogeneous population of poorly defined biologic inhibitors. In addition, a granulocyte chalone must be distinguished from products of mature neutrophils which influence the production of CSA but have little direct effect on CFU-C.³³⁻³⁴

Our original intent was to substantiate the existence of the granulocyte chalone by following closely the methods of Rytömaa and Kiviniemi, and Paukovitz. Our results lead to the conclusion that mature human granulocytes contain potent inhibitors of the growth and ³H-TdR uptake of some proliferating cells, but these are not true granulocyte chalones. It would appear that the inhibition in the ³H-TdR assay was not exclusively of granulocytic origin and that both monocyte and lymphocyte fractions have some inhibitory activity that is partially heat labile. It is also evident that the inhibitory activity of granulocyte extracts is not target organ specific, as stipulated by others. Erythroid precursors are more sensitive than granulocyte precursors to NDIF in thymidine uptake, mitotic inhibition, and clonogenic assays.

To our knowledge, these were the first studies utilizing both myelopoiesis and erythropoiesis assays to test the validity of the granulocyte chalone hypothesis. Lord and his colleagues³⁵ recently reported that extracts of rat granulocytes failed to inhibit mouse myeloid colony formation in vitro—a finding consistent with our own observations. The relative refractoriness of human lymphocytes to NDIF conforms to the findings of others.⁵⁻⁶ The absence of species specificity and the sensitivity of leukemic blasts to NDIF are in agreement with the findings of Rytömaa and Kiviniemi in regard to granulocyte chalone.

Although we have been unable to document that mature human granulocytes produce “chalone” in the strict sense of the word, we do suggest that they pro-
duce nonlethal potent inhibitors of cell replication that are more effective against erythroid precursors and are species nonspecific. The exact subcellular source, biochemical structure, mechanism of action, and physiologic significance of these inhibitors are presently under investigation.

ACKNOWLEDGMENT

The authors wish to thank Carol Le Fèvre for excellent technical support and several colleagues at UCLA for thoughtful review of the manuscript.

REFERENCES

23. Boyum A: Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifuga-
GRANULOCYTIC "CHALONE"


35. Lord BI, Testa NG, Wright EG, Banerjee RK: Lack of effect of a granulocyte proliferation inhibitor on their committed precursor cells. Biomedicine 26:163, 1977
Neutrophil products that inhibit cell proliferation: relation to granulocytic "chalone"

SP Herman, DW Golde and MJ Cline