Some Circulating Factors Which Influence Granulocyte-Monocyte Production in the Chick with Myeloblastic Leukemia

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ABSTRACT

Soft-agar cloning was used to investigate possible granulopoietic-monopoietic regulatory defects in the chick with myeloblastic leukemia induced by avian myeloblastosis virus. The plasma levels of granulocyte-monocyte colony-stimulating activity (CSA) of normal and leukemic plasmas were the same when undiluted or unfractionated plasmas were tested. However, dilution or fractionation revealed elevations in plasma CSA levels in the leukemic animals. Most of the activity in both normal and leukemic plasma eluted in the void-volume peak during Sephadex G-200 gel filtration, and the CSA levels in the leukemic peak were increased 5- to 10-fold. This increase did not reflect higher levels of an inhibitory lipoprotein eluting in the void volume since the differential between normal and leukemic plasma was present after delipidation. We therefore investigated the possibility that a nonlipoprotein inhibitor was present. Sephadex G-200 chromatography of leukemic plasma revealed that a potent inhibitor of colony formation was present in one of the peaks of material eluting from the column. Eight μg of this material inhibited colony formation by 50%. This inhibitory material was not detected in corresponding fractions obtained after chromatography of normal plasma. These data show that plasma from the chick with myeloblastic leukemia has markedly elevated levels of CSA and that it also contains an inhibitor of colony formation which is absent or present at very low levels in normal plasma. Finally, leukemic plasma contained abundant amounts of avian myeloblastosis virus polypeptides which are being investigated for possible relationships to the above-described activities.

INTRODUCTION

The granulocyte-monocyte progenitor cell replicates in semi-solid media to produce recognizable colonies if CSF3 is present (6, 27). In acute myeloblastic leukemia, one would expect the accumulation of nonmaturing myeloblasts to reflect a defect in granulopoietic regulation. In that context, decreased (28) as well as increased (22) levels of serum stimulatory activity have been reported in this disease. Others have reported increased (2) or unchanged (15) levels of serum stimulating activity for the avian model to determine: (a) the levels of stimulating activity in unfractionated normal and leukemic plasma; (b) their levels in fractionated normal and leukemic plasma; and (c) the contribution of inhibitory lipoproteins to the net stimulatory activity.

It should be stated parenthetically that we recently described 2 types of plasma stimulatory factors, only one of which stimulated colony formation directly (9). The second acted indirectly, probably by enhancing production of CSF in the assay. In this report, undefined stimulatory activity of plasma resulting from the combined effects of these factors, plus that of at least one type of inhibitor (certain lipoproteins), will be referred to as CSA. CSF will be used in reference to a purified molecule which, by itself, stimulates colony formation or to a CSF-type molecule(s) which has at least been separated from molecules that inhibit colony formation and from those which merely indirectly stimulate colony formation.

MATERIALS AND METHODS

Animals, Virus, and Plasma. Inbred chicks (line 6 of the Department of Agriculture, East Lansing, Mich.) were maintained in our facilities. Within 24 hr after hatching, chicks were

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§ The abbreviations used are: CSA, colony-stimulating factor; AMV, avian myeloblastosis virus; M.W., molecular weight; TBS, 0.025 M Tris/0.15 M HCl (pH 7.4); NPI, NPII, LPI, LPII, etc., gel filtration absorbance peaks (P) derived from normal (N) and leukemic (L) plasma.

P. Bentvelsen, personal communication.
inoculated i.v. with viremic plasma containing $10^4$ to $10^5$ transforming units of standard AMV (Bureau of Animal Industry, strain A). Transformation was assayed on marrow cell monolayers (24). Peripheral blood smears were made at weekly intervals (beginning at 2 weeks) and, when myeloblasts represented greater than 80% of the leukocytes (2 to 4 weeks postinfection), heparinized blood was obtained. A normal hatchmate was bled at the same time. Plasmas were stored at 4° and analyzed within a week.

**Gel Filtration.** Sephadex G-200 gel filtration was done in 0.05 M Tris-HCl buffer, pH 7.4, at 4°. Column dimensions were 2.6 x 50 cm (void volume, 60 ml; bed volume, 240 ml). Fractions (2 to 4 ml) were collected at timed intervals, sterilized by filtration, and stored at 4° until assayed (within 1 week). Normal and leukemic plasmas were chromatographed within the same week.

**SDS-PAGE.** This procedure was carried out as described previously (4). In general, samples were lyophilized, then dissolved in a solution of 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol and heated for 2 min at 100°. Bromophenol blue (0.02%) and glycerol (25%) were added to each sample, and each was placed onto a 10% polyacrylamide gel. Samples were electrophoresed at 35 mA and 70 V, for 5 hr, stained with 0.25% Coomassie blue in methanol:acetic acid:water (5:1:5), and destained with methanol:acetic acid:water (3:2:40) before photography.

**Double Immunodiffusion.** Analyses were carried out by placing test materials in the outside wells of immunodiffusion plates prepared with 1% agar and 0.85% NaCl solution and by placing specific antisera in center wells. A rabbit antisemir specific to avian serum albumin was purchased from Cappel Laboratories, Inc., Cochranville, Pa., and a goat antiserum (gp85) of AMV was purified and labeled with 125I as described previously (33). Rabbit anti-AMV gp85 was prepared as characterized previously (34). Briefly, increasing amounts of test samples were incubated (37° for 120 min) with a 1:1000 dilution of rabbit anti-AMV gp85 (an amount sufficient to precipitate 50% of labeled AMV gp85). A constant amount of 125I-labeled AMV gp85 (30,000 cpm) was then added, and incubation was continued for 90 min at 37°. Goat anti-rabbit serum was added, and the incubation was continued for 90 min at 37°, and overnight at 4°. Immunoprecipitates were then washed and counted in a gamma counter.

**Plasma Delipidation by Ultracentrifugation.** Total lipoproteins were removed from plasma by preparative ultracentrifugation ("floation fractionation"), as described by Havel et al. (14). Blood was collected in syringes containing 0.1 ml of 5% EDTA and chilled. Plasma was collected, and 10 ml were adjusted to a density of 1.225 g/ml with the addition of 3.517 g potassium bromide. The solution was transferred to an ultracentrifuge tube and an overlay of a 1.225-g/ml density solution (0.3517 g KBr per ml, 0.9% NaCl, and 5% EDTA) was gently added. This solution was then centrifuged at 200,000 x g for 40 hr in a swinging bucket rotor. The top of the tube containing lipoprotein was removed with a tube slicer and the infranatant was dialyzed against TBS prior to assay for CSA.

**Chloroform Extraction.** Chloroform extraction of serum was performed essentially as described by Granström et al. (13). Briefly, 1 volume of serum was mixed with 4 volumes of chloroform, shaken for 2 min, and incubated at 20° for 40 to 60 min. The aqueous layer was removed, centrifuged at 5000 x g for 20 min, and incubated at 4° for 12 hr.

** Colony Assay.** This assay was performed as described previously (8). In general, 10⁶ un fractionated or nonadherent marrow cells were suspended in 1.5 ml of Medium 199 supplemented with calf serum (10%) and tryptose-phosphate broth (15%) and containing Bacto agar (Difco Laboratories, Inc., Detroit, Mich.) at 0.36%. Nonadherent cells were obtained by an overnight plastic adherence step as described previously (8). After solidification, 0.15 ml of test material was gently added to the agar medium. Colonies (≥50 cells), clusters (10 to 49 cells), and miniclusters (3 to 9 cells) were enumerated at 10 days. All samples were assayed at least in duplicate and in parallel with a negative control fluid (TBS).

**Protein Determinations.** Protein concentration was determined by the "Bio-Rad procedure" (5).

**RESULTS**

**Analysis of Unfractionated Plasma.** Undiluted leukemic plasma did not differ appreciably from undiluted normal plasma in its ability to support cluster or colony formation by nonadherent cells (Table 1). Similar results were obtained with unfractionated cells. Dilution analyses routinely showed, however, that leukemic plasmas exhibited more stimulatory activity. A representative titration done on nonadherent cells is shown in Chart 1. The titer (last dilution supporting growth) of leukemic plasma was at least 2-fold greater than that of normal plasma and, at the last dilution tested (1:8), the activity of leukemic plasma was 10-fold greater than that of normal plasma. A comparison of normal and leukemic sera gave similar results.

**Analysis of Plasma Fractionated by Gel Filtration.** When plasmas were fractionated by Sephadex G-200 gel filtration and assayed on unfractionated bone marrow cells, marked differences were revealed in CSA levels. Most of the activity in both normal and leukemic plasmas was associated with a peak coinciding with the void volume of the column (Chart 2). However, the LPI stimulated approximately 1300 colonies plus clusters, while the NPI stimulated approximately 120 colonies plus clusters (note the scale change in Chart 2). Furthermore, the 3 most active fractions from LPI (Fractions 40, 42, and 43; protein concentration, 335 μg/ml) supported an average of 440 colonies, while the most active fractions from NPI (Fractions 20 and 22; protein concentration, 730 μg/ml) supported an average of 10 colonies. These results indicate that leukemic plasma contained a higher level of stimulatory activity than did normal plasma when fractionated sera were analyzed.

To further examine the stimulatory activity of fractionated plasmas, fractions of the different Sephadex G-200 "peaks" and "valleys" were pooled and tested on both unfractionated and nonadherent cells (Table 2). Three points were noteworthy: (a) LPI was much more active than was NPI (10-fold); (b) only PI's were active on nonadherent cells; and (c) most of the activity for unfractionated cells was also located in the PI's, but
Factors in Avian Myeloblastic Leukemia

Table 1

Plasma stimulatory activity for nonadherent cells

Heparinized plasmas were obtained from normal and frankly leukemic chicks, stored at 4°C for 3 to 6 days, and assayed (0.15 ml) on nonadherent marrow cells (1 x 10⁵). Plasmas derived from frankly leukemic and age-matched control animals are indicated by identical numbers. The control has the suffix of "C." Plasmas were collected from 3 experiments (1 to 3, 953 and 955, and 1049 to 1060) and tested on 3 different marrow populations.

<table>
<thead>
<tr>
<th>Plasma</th>
<th>Animal</th>
<th>No. of clusters</th>
<th>No. of colonies</th>
<th>Total (clusters + colonies)</th>
<th>Av. of totals</th>
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<tr>
<td>Frankly</td>
<td>1049</td>
<td>838 ± 19</td>
<td>214 ± 58</td>
<td>1052 ± 10</td>
<td>1216</td>
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<tr>
<td></td>
<td>1051</td>
<td>590 ± 0</td>
<td>275 ± 9</td>
<td>865 ± 10</td>
<td></td>
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<tr>
<td></td>
<td>1059</td>
<td>724 ± 206</td>
<td>288 ± 10</td>
<td>1012 ± 218</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1060</td>
<td>503 ± 9</td>
<td>94 ± 0</td>
<td>597 ± 9</td>
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<td></td>
<td>1</td>
<td>798 ± 161</td>
<td>281 ± 114</td>
<td>1079 ± 47</td>
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<tr>
<td></td>
<td>2</td>
<td>1079 ± 66</td>
<td>221 ± 123</td>
<td>1300 ± 190</td>
<td></td>
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<tr>
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<td>3</td>
<td>872 ± 170</td>
<td>100 ± 9</td>
<td>972 ± 161</td>
<td></td>
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<tr>
<td></td>
<td>953</td>
<td>1394 ± 170</td>
<td>663 ± 104</td>
<td>2057 ± 66</td>
<td></td>
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<tr>
<td></td>
<td>955</td>
<td>1246 ± 284</td>
<td>764 ± 0</td>
<td>2010 ± 264</td>
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<tr>
<td>Normal</td>
<td>1049C</td>
<td>657 ± 70</td>
<td>87 ± 67</td>
<td>744 ± 104</td>
<td>1259</td>
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<tr>
<td></td>
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<td>107 ± 19</td>
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<td></td>
<td>1060C</td>
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<td>898 ± 52</td>
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<td>1233 ± 233</td>
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* Mean ± S.D.

some activity was associated with the other peaks (primarily normal).

**Protein Composition of Sephadex G-200 peaks.** The absorbance profile obtained with leukemic plasma was often different from that obtained with normal plasma. The reason for this was not clear. The volumes chromatographed in each case were approximately equal, and the void volumes remained unchanged after each fractionation. To determine if there were major qualitative differences in the protein composition of normal and leukemic peaks, SDS-PAGE and double immunodiffusion analyses were done. The SDS-PAGE patterns of LPIII

![Chart 1. Titration of normal and leukemic plasmas. Heparinized plasma was obtained from a frankly leukemic chick and a normal hatchmate, stored at 4°C within 1 week on nonadherent marrow cells (1 x 10⁵).](chart1)

![Chart 2. Fractionation of normal and leukemic plasma by gel filtration. Normal and leukemic plasmas were stored at 4°C for 3 days and then chromatographed on Sephadex G-200 at 4°C in 0.05 M Tris-HCl, pH 7.4 (similar results were obtained with fresh plasmas). Alternate fractions were tested for CSA on 1 x 10⁵ unfractionated marrow cells (note the change of scale on right ordinate). The plasmas were chromatographed within the same week and tested on the same marrow 1 day after fractionation of the second plasma. A, chromatogram of normal plasma; B, chromatogram of leukemic plasma.](chart2)

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and NPIII were very similar (Fig. 1a). Both peaks contained one major protein which comigrated with chicken serum albumin (Cappel Laboratories, data not shown) and contained material which was immunoprecipitated with monospecific antichicken serum albumin (Fig. 2a). Likewise, the SDS-PAGE patterns of LPII and NPII were similar (Fig. 1b). One of the major proteins (at the bottom in each peak) comigrated with the light chain of chicken IgG (Cappel Laboratories, data not shown), and material in this peak reacted with monospecific goat antibody to chicken IgG (Fig. 2b). Finally, the peptide patterns obtained from LPI and NPI were very similar (Fig. 1). Since LPIII and NPIII apparently shared at least 3 peptides, and LPI and NPII apparently shared at least 11 peptides (in each case, the shared peptides accounted for the majority of the peptides); and since the immunoprecipitation data showed that the major marker proteins of NPI and NPIII were also present in LPII and LPIII, we concluded that LPII and LPIII corresponded to NPII and NPIII even though they eluted differently from NPII and NPIII.

Some peptides were unique to either the normal or the leukemic peaks. For instance, 1 to 2 peptides of low molecular weight were present in each normal peak but absent from the corresponding leukemic peak. Leukemic peaks (LPI, LPIII) contained 2 to 3 unique peptides (usually minor components) and some of these comigrated with structural peptides of AMV. One of the unique peptides in both LPI and LPIII comigrated with AMV structural protein, M.W. 27,000, the major structural polypeptide of the virion. This was a major component in LPI.

Some experiments were done to determine whether the low activity of NPI relative to LPI was due to higher levels of inhibitory lipoproteins in NPI. In the first experiment, lipoproteins were removed from a pair of normal and leukemic plasmas by ultracentrifugation in potassium bromide. The resulting aqueous phase was dialyzed against TBS and fractionated by Sephadex G-200 chromatography. PI fractions were then pooled and assayed on nonadherent cells. The amount of growth per clone was less than usual due to the 2 purification steps, but it was evident that LPI was more active than NPI (at least 4-fold). LPI supported 2231 ± 426 (S.D.) miniclusters and 54 ± 0 clusters while NPI supported only 529 ± 256 miniclusters.

In another experiment, a different pair of normal and leukemic plasmas was chromatographed by Sephadex G-200 chromatography. The resulting PI's were extracted with chloroform and tested on unfractionated cells. The numbers of clones supported by LPI before and after extraction were 188 ± 0 and 151 ± 1. The numbers supported by NPI were 34 ± 5 and 0. The absence of activity in the extracted NPI sample is not understood and is being investigated. The main point of interest related to the current study, however, is that delipidation did not increase NPI activity to the level of LPI. Therefore, the differential observed between NPI and LPI was not due to different levels of inhibitory lipoproteins.

**Inhibitor in Leukemic Plasma.** Since diluted or fractionated leukemic plasma exhibited higher levels of CSA in contrast to undiluted or unfractionated leukemic plasma, we postulated the existence of an inhibitor in leukemic plasma. This inhibitor presumably would not be lipoprotein since LPI made from delipidated plasma still exhibited higher CSA levels than NPI and since these inhibitory molecules would elute in the Sephadex G-200 void-volume PI wherein the differential in CSA levels was apparent. LPII and LPIII were obvious regions to test as they exhibited no CSA. An inhibitor was detected in LPII (Chart 4). Eight µg (1:10 dilution) of this material blocked colony formation by 50%. Inhibitory activity was not detected in other fractions of leukemic plasma or in corresponding fractions of normal plasma.

**DISCUSSION**

Circulating CSA levels in leukemic chicks were markedly elevated (5- to 10-fold). This increase was not related to lower levels of inhibitory lipoproteins inasmuch as it was apparent...
regardless of whether plasmas had been delipidated. This differential in CSA levels, however, was noted only after dilution or fractionation of the plasma. These observations suggested the presence of a nonlipoprotein inhibitor in leukemic plasma, and we provide evidence for the existence of a nonlipoprotein inhibitory molecule. Preliminary findings indicated the following characteristics: heat stability (65°, 30 min); acid-stability (pH 2, 30 min); low molecular weight (<20,000) as determined by high-performance liquid chromatography and a direct antimitotic effect. Others also detected a leukemia-associated inhibitor in the plasma of acute myeloblastic leukemia and acute lymphocytic leukemia patients (23). Future studies will be aimed at identifying relationships to previously described granulopoietic inhibitors, prostaglandin E (16), lactoferrin (20), granulocyte chalone (18, 29, 30), interferon (19), and leukemic inhibitory activity (7).

Most CSA in both normal and leukemic plasma eluted in the void volume during Sephadex G-200 chromatography. The same observation was made with chick endotoxemic plasma (9). Preliminary data revealed, however, that the molecular weight of partially purified endotoxemic plasma CSF was approximately 20,000 (determined by SDS-PAGE). Others noted that the apparent molecular weight became smaller with increasing purity (32).

Some activity was detected in the other Sephadex G-200 peaks but only when unfractionated cells were used. This type of activity was also detected in the included volume of Sephadex G-200 when human plasma was chromatographed (1). Indeed, several groups have noted that most plasma CSA is adherent cell dependent (1, 3, 9, 12). In our hands, activity for nonadherent cells was also detected, but it was associated only with the excluded volume of the Sephadex G-200 chromatogram. Such activity has not been detected in human plasma (1). However, the much higher levels of inhibitory lipoproteins in the human, relative to the chick [human, 82% in plasma (12); chick, 25% in plasma]5 may have precluded detection of this activity since they would also elute in the void volume. Indeed, the excluded volume material derived from human plasma not only did not exhibit CSA; it was inhibitory (1).

In summary, we believe that the chick with myeloblastic leukemia has at least 2 defects in hemopoietic regulation: (a), circulating levels of CSA are markedly elevated; and (b), an inhibitor not normally present appears. Future studies will be aimed at characterizing these factors and determining whether they are related to leukemic progression.

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Fig. 1. Polyacrylamide gel electrophoresis of chromatographic fractions obtained from normal and leukemic plasma. Forty µg of each of the different Sephadex
G-200 absorbance peaks were lyophilized, dissolved in SDS and 2-mercaptoethanol, electrophoresed, and stained as described in “Materials and Methods.” Arrows,
position (from top to bottom) of AMV polypeptides (4), gp65, p27, p12, and chalone which were coelectrophoresed.

Fig. 2. Double immunodiffusion analysis of chromatographic fractions. Pooled fractions of the Sephadex G-200 peaks were assayed for marker proteins by
immunodiffusion. a: center well, 20 µg IgG fraction of rabbit anti-chicken serum albumin (Cappel Laboratories); outside wells: Well A, normal chicken serum (12.5
µg); Well B, LPI (6 µg); Well C, LPIII (7 µg); Well D, LPII (5 µg); Well E, NPI (4 µg). b, center well, 25 µg goat anti-chicken IgG; outside wells as in a.
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