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Myeloid Leukemic Cell Differentiation Induced by Human Postendotoxin Serum and Vitamin Analogues *

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A. Introduction

Fibach and Sachs [11] reported that serum from mice injected with endotoxin induced granulocyte and macrophage differentiation of the mouse myeloid leukemic cell line M1. At that time it was unclear whether the differentiation-inducing factor was CSF (MGI-1). Metcalf [28] also reported that a pure preparation of GM-CSF has some capacity to induce differentiation of murine WEHI-3 myelomonocytic leukemic cells. More recently biochemical characterization of postendotoxin serum has shown that the differentiation factor could be separated from GM-CSF (MGI-1) [24] and was termed MGI-2, or could be separated from the bulk of serum CSF but coeluted with a minor species of CSF that stimulated only granulocyte colony formation [5].

The cellular origin of the differentiation factor (DF) for leukemic cells is diverse (Table 1). Spleen lymphocytes and macrophages have been shown to produce the activity when treated with various mitogens including endotoxin [45]. Conditioned medium from a variety of murine organs also contained DF, but much higher levels of activity were reported following endotoxin treatment [34]. DF produced by the different organs in vitro and found in vivo in endotoxin serum was distinguishable from the majority of granulocyte-macrophage (GM) and macrophage (M) CSF. DF was precipitated by 55% ammonium sulfate and did not bind to Concanavalin A-Sepharose whereas CSF required 85% saturation and bound to Con A-Sepharose. DF also eluted later than CSF from phenyl-Sepharose columns and could be distinguished form M-CSF by its apparent molecular weight (23,000) on native gels [34].

Introduction of DF is radioresistant and independent of T cells since nude mice respond normally [28]. Repeated injections of endotoxin lead to depressed responsiveness as measured by serum DF and CSF levels, and sustained serum DF levels have not been obtained [28]. This is in part due to the short serum half-life of GM-DF (1.5–3 h) and the development of endotoxin "tolerance," which provides a possible major obstacle to the clinical value of endotoxin induction of endogenous mediators.

Endotoxin or other immunostimulants from microorganisms may also induce leukemic cell differentiation in vitro by inducing the leukemic cells to produce endogenous. DF [2, 10, 43]. The structure of micoroorganisms required for a direct effect on the differentiation of leukemic cells and for stimulation of DF production by normal spleen cells may differ since BCG induced direct differentiation of M-1 leukemic cells and spleen DF production, *C. parvum* had no direct effect on M-1 cells but was a potent inducer of spleen cell DF production and *N*-acetylmuramyl dipeptide, and minimal adjuvant-active subunit of the bacterial cell wall had no direct effect on the differentiation of M1 cells and only slightly stimulated the production of D-factor by spleen cells [45].

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Table 1. Sources of GM-DF (MGI-2, D-factor)

Mouse	
Postendotoxin serum	Fibach and Sachs [11], Metcalf [27], Lotem et al. [24], Burgess and Metcalf [5]
Normal mouse organ	Nicola and Metcalf [34]
Lung CM	Maeda and Sachs [25], Metcalf [26], Lotem et al. [24]
Embryo and fibroblast CM	Ichikawa [20], Fibach and Sachs [12], Hayashi et al. [17]
Macrophages	Ichikawa [20], Hozumi et al. [19], Ichikawa et al. [21], Lotem et al. [24], Yamamoto et al. [45]
Stimulated spleen lymphocytes	Hozumi et al. [19], Lotem et al. [24], Akagawa et al. [3], Yamamoto et al. [45]
T-cell hybridoma	Burgess et al. [6]
Ascites from tumor-bearing mice	Hozumi et al. [19], Hayashi et al. [45]
Tumor cell lines	Lipton and Sachs [23], Sachs [41], Yamamoto et al. [45], Moore [30]
Human	
PHA; PWM leukocyte CM	Elias et al. [9], Olsson and Olofsson [35], Olsson et al. [36]
Amniotic fluid	Nagata et al. [33]
Placental CM	Olsson and Olofsson [35]
Postendotoxin serum	Moore et al. [32]

Endotoxin-containing bacterial vaccines have been reported to have therapeutic activity in patients with actue myeloblastic leukemia and nodular lymphoma [22]. In an early study at the Memorial Sloan Kettering Cancer Center conducted from 1970 to 1972, patients with acute myeloblastic leukemia were randomized to receive a vaccine pepared from *Pseudomonas aeruginosa* in addition to chemotherapy protocol. The vaccine was a soluble extract which contained much endotoxin and was intended to prevent or ameliorate *Pseudomonas* infection which pose a serious threat to patients with acute leukemia. While the frequency and severity of *Pseudomonas* infections was not affected by the vaccine, the duration of remission was much longer in the vaccinated group. Six of 13 patients who achieved remission in the vaccine group and 1 of 17 patients in the no-vaccine group are still in complete remission 9 years later. Mixed bacterial vaccine in conjunction with chemotherapy in patients with nodular non-Hodgkins lymphoma also resulted in a higher rate of complete remission, longer duration of remission, and highly significant longer survival [22].

It has become clear that many of the diverse biological effects of endotoxin are not caused by endotoxin itself but by endogenous mediators released by host cells in respone to endotoxin. Three mediators of possible relevance to the antileukemic effects of endotoxin are tumor necrosis factor (TNF), Interleukin-1 (IL-1), and leukemia-differentiating factor (DF); in addition endotoxin is a potent inducer of prostaglandins, interferons, and various species of myeloid colony stimulating factors.

Vitamin A and its analogues (retinoids) also affect proliferation and differentiation of normal and malignant hematopoietic cells. The most extensively studied system has been the human promyelocytic leukemic cell line HL-60 which can be induced to differentiate to mature granulocytes (as measured morphologically, by differentiation antigen expression, and functionally by ability to reduce nitro-blue tetrazolium), following exposure to retinoic acid [4, 18, 36, 38]. Maximum differentiation (approximately 90% of cells) occurs

with 1 μM retinoic acid, a concentration 500- to 160,000-fold less than the concentration of butyrate and dimethyl-sulfoxide that promotes a similar increase in differentiation. Continous exposure to retinoic acid is necessary for maximum differentiation, which occurs after 5 days of incubation, and retinol (vitamin A), retinal acetate, and retinal are approximately 1000-fold less potent than retinoic acid, which can induce some differentiation at concentrations as low as 1 nanomole.

Retinoid induction of myeloid leukemic differentiation is not a universal phenomenon. While the murine myelomonocytic leukemic cell line WEHI-3 can be induced to mature neutrophil differentiation [31] and retinoic acid induces the human malignant monoblast line U937 to monocyte-like cells with the capacity to reduce nitroblue tetrazolium [36], the human myeloid cell lines KG-1 and K562 cannot be induced to differentiate [7]. The mouse myeloid leukemia, M1, can be induced to increase levels of lysomal enzyme production without induction of phagocytosis, locomotive activity, or morphological maturation [43]. Indeed retinoic acid was a potent inhibitor of induction of these latter differentiation-associated properties. Fresh leukemic cells from patients with various myeloid leukemias have also been exposed to retinoic acid in short-term primary suspension cultures and morphological and function maturation was observed only in cases of acute promyelocytic leukemia [14].

The active form of vitamin D_3, 1 alpha, 25-dihydroxyvitamin D_3, and vitamin D analogues may also prove of clinical utility in inducing myeloid leukemic cell differentiation. Abe et al. [1] theorized that since the active form of vitamin D_3 causes multinucleate osteoclasts to appear in bone resorbing surfaces and osteoclasts are thought to be derived from monocytes and macrophages then the murine myeloid leukemia cell line M1 would be a good model to test whether vitamin D can induce this cell line to differentiate into macrophages. The degree of leukemic cell differentiation induced by 12 nM 1 alpha 25-dihydroxyvitamin D was comparable to that induced by 1 nM dexamethasone, the most potent known stimulator of M1 differentiation, and unlike the action of retinoic acid, differentiation-induction included development of phagocytic ability, receptor expression, and locomotive activities. In addition, the vitamin markedly inhibited cell growth in a time-dependent manner. Myelopoietic stimulatition by vitamin D in vitro has been reported by Salahuddin et al. [42], who described a method for the routine long-term growth (greater than 3 months) of normal immature human myeloid cells in liquid suspension culture. The technique employs cell-separated cord blood leukocytes and special growth conditions including hydrocortisone and an obligatory requirement for vitamin D. The augmentation of normal myelopoiesis and inhibition of myeloid leukemic cell proliferation with induction of differentiation seen at comparable in vitro concentrations of vitamin D_3 suggest that in vivo studies may be warranted, particularly since calciferol induces differentiation of leukemic cells refractory to retinoic acid (e. g., M1) and may have significantly fewer side effects at comparable dose levels.

B. Materials and Methods

CFU-c assay: Bone marrow aspirates were obtained from normal volunteers and patients after informed consent. Marrow cells were allowed to sediment, and the leukocyte-rich plasma was collected, centrifuged, and the cells washed twice prior to in vitro culture at 1×10^5 cells/ml in 1.0 ml 0.3% agar in McCoys modified medium containing 10% fetal calf serum. Cultures were stimulated by addition of 10% (v/v) of human GCT cell line conditioned medium (Gibco) as a source of CSF. Cultures were scored at 7 days and the incidence of colonies of more than 40 cells and of clusters of 3–40 cells recorded. Mouse bone marrow cultures of 5×10^4 Balb/c cells were also established in McCoys-agar medium in the presence of WEHI-3 conditioned medium or L-cell conditioned medium as a CSF source.

Leukemic cell lines: Two continuous cell lines of the Balb/c murine myelomonocytic leukemia WEHI-3 were used as murine leukemic targets – the first, reported by Ralph et al. [39] was derived from the 125th

in vivo passage of the B subline of WEHI-3 and is not inducible to differentiate but has the capacity to produce a spectrum of cytokines, particularly many species of CSF [29]. A second cell line was independently developed from WEHI-3B at an early stage of in vivo passage and can be induced to terminal granulocyte and/or macrophage differentiation with loss of self-renewal capacity [26, 30]. To distinguish this line from that of Ralph et al. [39], we have adopted the nomenclature WEHI-3B D^+ for the former and D^- for the latter non-differentiating line. Two cloned cell lines of the human promyelocytic leukemia HL-60 (kindly provided by Dr. R. Gallo, NCI) were used as human leukemic targets. One, termed HL-60 D^-, was originally obtained in 1978 and after prolonged passage lost the capacity to undergo differentiation in response to a variety of inducing agents. The second line, HL-60 D^+, was more recently obtained from a cryopreserved early passage of the original HL-60 and retains neutrophil, eosinophil, and macrophage differentiation potential.

For assay of differentiation induction, titrated serum samples or vitamin analogues were added to 1.0-ml cultures (three plates per point) of 0.3% agar (Difco) in McCoy's modified medium containing 10% fetal calf serum and 300 WEHI-3B, or 1000 HL-60 leukemic cells. Cultures were incubated for 7 days in the case of WEHI-3, or 11–14 days with HL-60 cells, and scored for total number of colonies and total differentiated colonies. Differentiation was characterized by conversion of colonies from compact to diffuse [26]. Diffuse or dispersed colonies reflected differentiation of colony cells to mature neutrophils or macrophages with migratory properties. Additional confirmation of colony differentiation was provided by isolation of individual colonies by pipette, their transfer to glass slides followed by staining with aceto-orcein and microscopic examination.

Suspension cultures of HL-60: HL-60 cells were established in suspension culture at an initial concentration of 5×10^5 cells/ml in 10 ml modified McCoy's medium and 10% fetal calf serum. 13 *cis*-retinoic acid at 10^{-6}–10^{-7} or 1,25-dihydroxycholecalciferol at concentrations from 0.001 μg to 10.0 μg/ml were added at the initiation of the cultures. Following incubation, cultures were assayed at 24 h and 4 and 7 days for total cell count, morphology, and colony-forming capacity upon cloning in agar in the absence of vitamin analogues.

Vitamin analogues: 13-cis retinoic acid and 1,25-dihydroxycholecalciferol were kindly provided by Dr. L. Itri, Hoffmann-La Roche. Analogues were dissolved in absolute ethanol (1 mg/ml) and if not used immediately, were stored at –70 °C in dark vials under nitrogen-saturated conditions.

Human postendotoxin serum: In conjunction with a phase I clinical trial of highly purified endotoxin from *S. abortus* (Novo Pyrexal-Westphal) administered to patients with advanced nonhematopoietic malignancy, we were able to monitor serum levels of CFS and GM-DF. Patients received i.v. injections of endotoxin on a biweekly schedule with escalting doses from 0.1 to 30 mg/m². Bone marrow and serum samples were obtained immediately preceding endotoxin injection and serum at 0.5, 2, 4, 6, 8, and 24 h thereafter. Serum samples were assayed at 10% and 1% v/v against target populations of WEHI-3, HL-60, or autologous marrow cells cloned in agar.

C. Results

Action of vitamin analogues on normal and leukemic cells in agar culture: 13 cis retinoic (RA) and 1,25-dihydroxycholecalciferol (calciferol) were added at concentrations of 10^{-6}–10^{-7} *M* to 1 ml agar cultures containing 1000 HL-60 D^+ leukemic cells, 300 WEHI-3 D^+ leukemic cells, or 10^5 normal human marrow cells stimulated by GCT-conditioned medium. As shown in Table 2, the cloning efficiency of HL-60 cells was 30%, and this was significantly reduced in the presence of either RA or calciferol. Differentiation, as measured by conversion of leukemic colonies from compact to diffuse, was extensive at both molar concentrations of both analogues and approached 100% of colonies at the higher concentration of calciferol. Morphological analysis of individually isolated colonies obtained at 11–14 days revealed that all the control HL-60 D^+ colonies remained undifferentiated blast/promyelo-

Table 2. Comparison of 13-*cis* retinoic acid and 1,25-dihydroxycholecalciferol in the cloning capacity and differentiation of leukemic cell lines and normal human bone marrow

Stimulus	HL-60 D^+		WEHI-3 D^+		Human marrow
	cols/10^3	(% differentiated)	cols/300	(% differentiated)	cols/10^5
Control	307	(0)	60	(9)	179
Calciferol 10^{-7} *M*	143	(45)	39	(55)	363
Calciferol 10^{-6} *M*	63	(91)	16	(83)	442
RA 10^{-7} *M*	237	(77)	56	(48)	194
RA 10^{-6} *M*	186	(87)	62	(93)	227

Table 3. Morphology of HL-60 promyelocytic leukemic colonies exposed to 13-*cis* retinoic acid or 1,25-dihydroxycholecalciferol

Stimulus	Total colonies	% control	% diffuse	Colony Morphology[a]			
				Blast/ prom.	PMN	Macro-phage	G/M mixed
PBS	307	(100)	2	100	0	0	0
10^{-6} *M* retinoic acid	186	(61)	87	0	43	16	41
1 μg calciferol	37	(12)	100	0	40	33	27

[a] Colony morphology determined on 75 individually isolated colonies following orcein staining

cyte in type whereas all diffuse colonies obtained following RA or calciferol exposure were composed of band segmented neutrophils, macrophages, or a combination of both differentiated cell types (Table 3). WEHI-3 D^+ cells had a 20% cloning efficiency, with 9% of these colonies showing some conversion to diffuse morphology in control cultures scored after 7 days (Table 2). RA was only slightly inhibitory to cloning but induced a significant degree of differentiation and conversion of leukemic colonies to diffuse type. Comparable concentrations of calciferol were significantly more effective in inhibiting leukemic colony formation and in inducing WEHI-3 D^+ differentiation (Table 2). In this experiment up to 2.5-fold enhancement of normal human marrow colony formation (exceeding that observed with optimal concentrations of CSF) was seen following addition of calciferol, and a lesser but still significant enhancement was seen with RA. Neither RA nor calciferol was capable of stimulating normal colony formation in the absence of a CSF source. The degree to which RA or calciferol enhanced normal human or mouse colony formation has proved variable from experiment to experiment, and in some cases no augmentation was seen; however, suppression of leukemic colony formation, particularly that produced by calciferol, has been reproducible. Calciferol appeared to be more potent than equivalent molar concentrations of RA in selective inhibition of primary cloning of differentiation-inducible leukemic cell lines. In a further experiment, calciferol (1 μg/ml) was tested for its capacity to inhibit proliferation as measured by colony inhibition, of the nondifferentiating D^- lines of HL-60 and WEHI-3. Only slight inhibition of human and mouse marrow myeloid colony formation (14% and 38% respectively) was seen at this concentration of calciferol (Table 4) whereas the WEHI-3 D^+ line was inhibited 83% and cloning of the WEHI-3 D^- line was still more sensitive, being inhibited 99%. Comparable results were obtained with HL-60 with 99%

Table 4. Comparison of D^+ and D^- leukemic cell sensitivity to colony inhibition by 1,25-dihydroxycholecalciferol

Calciferol 1 μg/ml	WEHI-3 (D^+)		WEHI-3 (D^-)		HL-60 (D^+)		HL-60 (D^-)		Human BM	Mouse BM
	cols/ 300	% diff.	cols/ 300	% diff.	cols/ 10^3	% diff.	cols 10^3	% diff.	cols/10^5	cols/10^5
Control	247	(4)	240	(0)	822	(0)	402	(0)	70	73
Calciferol	41	(73)	3	(0)	11	(100)	0	(0)	60	45

inhibition of cloning of the D^+ line and complete growth inhibition of the D^- (Table 4).

Action of calciferol and RA on suspension cultures of HL-60 cells: In confirmation of numerous earlier studies, the addition of RA at 10^{-7} *M* to suspension cultures of HL-60 D^+ cells initiated at a concentration of 5×10^5 cells/ml resulted in induction of neutrophil differentiation with 26% of cultured cells being differentiated granulocytes by day 6 of incubation in contrast to only 3% in control cultures. No monocyte or macrophage differentiation was seen in RA-stimulated cultures. In contrast, suspension cultures of HL-60 D^+ cells exposed to concentrations of calciferol from 10.0–0.001 μg/ml did not show neutrophil differentiation but did convert to cells of macrophage type. As shown in Fig. 1, calciferol at all concentrations suppressed approximately two to three fold the number of cells recovered after 7 days incubation, but of greater significance was the observation that of the cells recovered by 7 days, 99% were macrophages in the presence of 1 μg/ml calciferol, and even at concentrations of 0.001 μg/ml 78% were macrophages. No neutrophil differentiation was induced. Upon replating 7-day calciferol-exposed HL-60 cells in semisolid agar at 500 cells/ml, in the absence of secondary exposure to calciferol, a major inhibition of recloning capacity was seen (Fig. 1). In

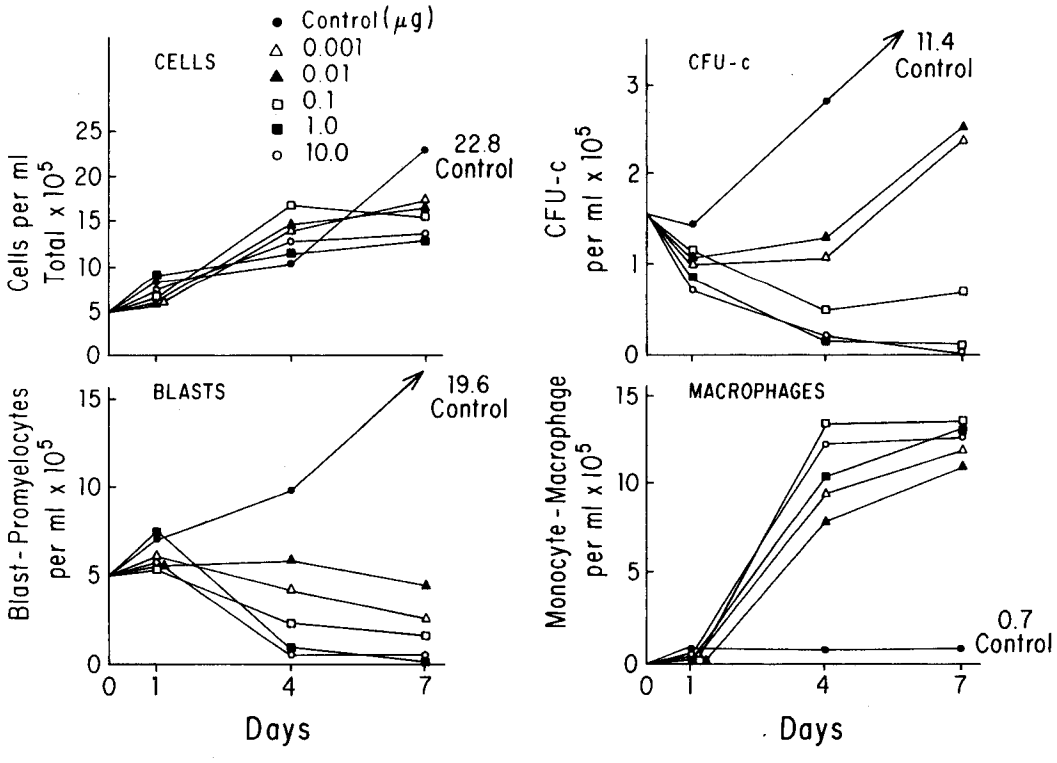

Fig. 1. Ten-milliliter suspension cultures of HL-60 D^+ leukemic cells at a concentration of 5×10^5 cells/ml were exposed to 0.001–10.0 μg 1,25-dihydroxycholecalciferol. At intervals of 1, 4, and 7 days cultures were assayed for cellularity, morphology, and recloning capacity in agar (CFU-c)

contrast to control cultures, which exhibited a 50% recloning capacity, almost complete (99%+) suppression of recloning was seen with HL-60 cells exposed to 1–10 μg/ml of calciferol, and at the lowest concentration of calciferol used (0.001 μg/ml) there was still a 70% inhibition. Colonies that did develop upon recloning of calciferol-treated HL-60 cells were compact and undifferentiated when cultured in the absence of secondary addition of calciferol.

Induction of a leukemia differentiation-inducing protein GM-DF in postendotoxin sera: A highly purified lipopolysaccharide prepared from *Salmonella abortus equi* in a sodium salt form (Novo-Pyrexal) was compared with conventional *E. coli* endotoxin (Difco) for capacity to induce GM-DF in mouse serum following intravenous injection. High titers of GM-DF were induced, peaking between 1.5 and 3 h post-injection with both types of endotoxin. Novo-Pyrexal was somewhat more potent than *E. coli* endotoxin, with detectable GM-DF induction at 0.1 μg. Following a dose of 5 μg/mouse, 3-h postendotoxin sera could be titrated to 1 : 640/1 ml culture of WEHI-3 D^+ cells and still induce leukemic cell differentiation significantly above background, and at a 1 : 20 serum dilution 100% of leukemic colonies were converted to diffuse in gross morphology and differentiated neutrophils and/or macrophages. No inhibition of leukemic colony formation was noted regardless of whether colonies were predominantly diffuse differentiated or compact in morphology.

In conjunction with a phase I clinical trial of Novo-Pyrexal in 25 patients with recurrent or metastatic cancer we were able to assess the capacity of endotoxin in graded doses to induce a human GM-DF in the patients' serum. Novo-Pyrexal was administered by intravenous injection, at doses ranging from 0.002 μg/m² to 55 μg/m². Nineteen patients received multiple doses, and 13 received doses higher than 1 μg/m². No differentiation-inducing activity against either WEHI-3 or HL-60 cells was seen in patient sera collected immediately prior to endotoxin injection, but within 30 min postinjection, a GM-DF activity was detected against both murine and human leukemic cells. The activity peaked between 2 and 6 h and was generally undetectable by 24 h (Fig. 2). At peak activity, up to 90% of WEHI-3 colonies and 50% of HL-60 colonies were induced to differentiate by a 1:10 dilution of the human postendotoxin serum, and GM-DF activity was still detectable at serum dilutions of 1:200. While GM-DF activity in mouse postendotoxin serum was higher than in human, it should be noted that maximum mouse activity was seen following injection of 200 μg endotoxin/kg whereas the human serum activity was obtained following injection of approximately 0.02 μg endotoxin/kg.

The ability of endotoxin to induce CSF in the serum of patients was confirmed by the assay of CSF on the patients' own bone marrow obtained prior to endotoxin injection. As previously reported, normal pre-endotoxin sera failed to stimulate normal myeloid colony formation, but CSF activity was detected 30 min postendotoxin and reached peak levels (272 ± 37 colonies per 10^5 marrow cells) 4–8 h postendotoxin treatment, returning to baseline activity by 12 h.

The reproducibility of endotoxin induction of GM-DF in human serum is illustrated in Fig. 2, which shows the results of injection of 1 μg/m² of endotoxin in five patients with advanced cancer. Induction of WEHI-3 differentiation was particularly marked, with all sera obtained 2–8 h post-endotoxin. An inhibitory activity was also noted in postendotoxin sera, appearing in sera collected within 30 min of endotoxin injection. This inhibitory activity led to reduction in WEHI-3 cloning but did not correlate with the kinetics of induction of GM-DF. An in vivo response to endotoxin was also seen in all patients since the white cell count invariably showed an acute decline within the first 2 h following endotoxin, then proceeded to return to normal levels or to a mild leukocytosis by 24 h (Fig. 2).

Development of pyrogenic tolerance after repeated administration of endotoxin was variable, and in some patients development of tolerance to the leukopenia-leukocytosis effect was also seen. Escalation of endotoxin dosage was effective in overcoming tolerance and biweekly administration of endotoxin to a maximum of 30 μg/m² produced consistent reinduction of serum GM-DF.

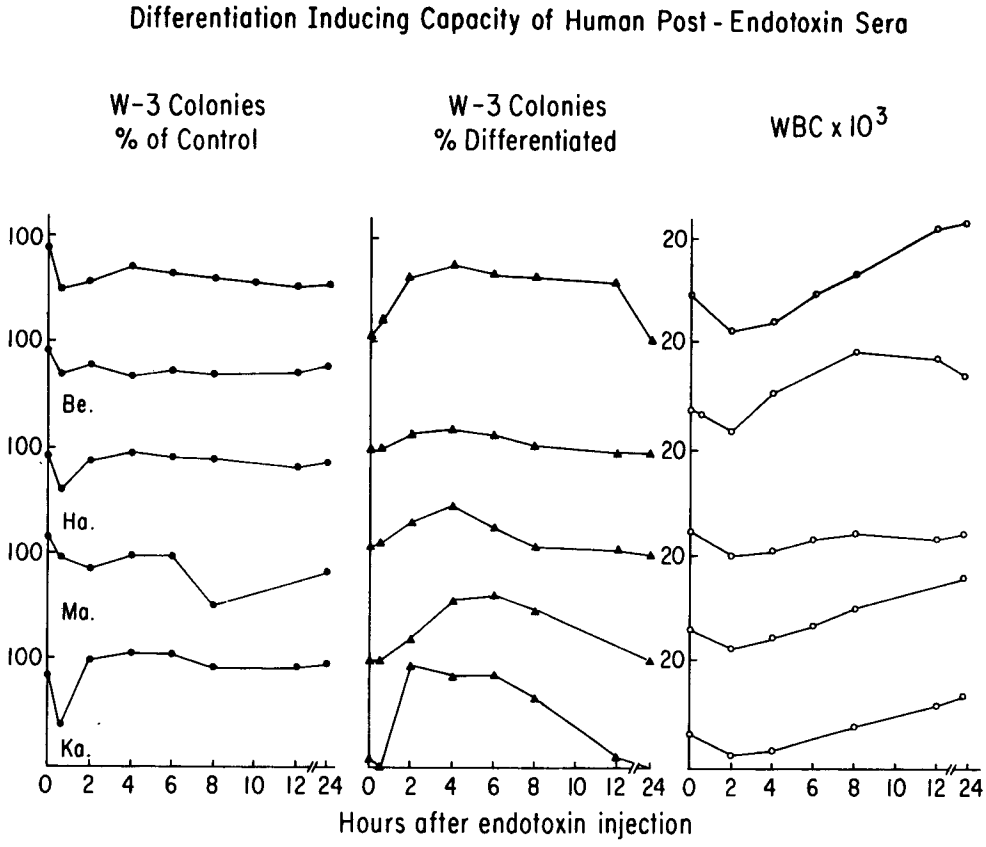

Fig. 2. Induction of a leukemia differentiation inducing factor (GM-DF) in the sera of five patients with advanced cancer following i.v. injection of 1 μg endotoxin (Novo-Pyrexal)/m². Serum DF assayed on a target population of WEHI-3B (D^+) leukemic cells. Note peak induction of differentiation factor 2–4 h postendotoxin. Serum colony inhibitory activity (*left panel*) was maximal 30 min after endotoxin (note, 1–10 μg of endotoxin added directly to cultures of WEHI-3B did not inhibit cloning nor induce differentiation)

D. Discussion

The evidence for a defined endogenous biological entity (GM-DG) with proven capacity to induce, in vitro, the differentiation of a variety of leukemic cell lines and suppress their in vivo leukemia-inducing potential is well established in murine experiments. The present report documents the inducibility of a similar human GM-DF in patients receiving endotoxin. Our ability to reproducibly induce human GM-DF following repeated escalating doses of endotoxin could provide the basis of a differentiation therapy in those leukemias where preliminary in vitro studies suggest a sensitivity to GM-DF. Ultimately, chronic-elevated levels of GM-DF could be maintained by administration of an exogenous source of the factor rather than depending upon injection of an inducer of endogenous activity. There is, however, some evidence to suggest that the multiplicity of endogenous cytokines induced by endotoxin may act synergistically. For example, our studies have shown the endotoxin induces a serum factor which inhibits leukemic cell cloning and differs from GM-DF. Furthermore, the endotoxin protocol induces elevated levels of GM-CSF and Interleukin I, which presumably would be effective in accelerating regeneration of normal myelopoiesis and immune function – particularly following chemotherapy-induced suppression.

The differentiation-inducing ability of retinoic acid has already promoted its use in phase I clinical trials in promyelocytic leukemia and pre-leukemic states [15]; however, antiproliferative action of retinoids upon leukemic cells is both more general than the incidence of induction of terminal differentiation and is seen with retinoid concentrations readily attainable in in vivo. The potential efficiency of retinoic acid in the treatment of human leukemia is further suggested by the observation that

retinoic acid enhances colony-stimulating factor-induced clonal growth of normal human myeloid progenitor cells in vitro [8]. Maximal stimulation occurred at a concentration of $3 \times 10^{-7} M$ retinoic acid, which increased the mean number of colonies by $213 \pm 8\%$ over plates containing CSF alone. Retinoic acid has no direct CSF activity nor does it stimulate CSF production by the cultured bone marrow cells. This stimulation may be mediated by increased responsiveness of the granulocyte-macrophage progenitors and the action of CSF, possibly by increasing the number of CSF receptors per cell.

The ability to sustain chronic levels of retinoic acid in patients with only minor toxicity suggests that this may be a valuable adjunct either to conventional chemotherapy or to additional forms of biological response modification. In this context, leukemia differentiation-inducing factor (GM-DF) produced by mitogen-stimulated human leukocytes acts synergistically with retinoic acid in inducing maturation of the human leukemic lines U-937 and HL-60 [36], and compounds elevating intracellular levels of cAMP, such as dibutryl cAMP, prostaglandin E, and choleratoxin acted synergistically with retinoic acid to induce maturation of both cell lines.

The potential therapeutic value of the active metabolite of vitamin D_3 – 1,25-dihydroxycholecalciferol – in leukemia is supported by our observations on the in vitro response of both D^+ and D^- clones of WEHI-3 and HL-60 cells. In contrast to the action of retinoic acid, calciferol favors a macrophage rather than granulocyte pathway of leukemic cell differentiation, with extinction of leukemic cell self-renewal. In this context, it mimics the macrophage-inducing action of tumor-promoting phorbol diesters [40] and may share the broad spectrum of leukemic cell types responsive to phorbol ester-induced differentiation [37]. In addition to inducing differentiation, low concentrations of calciferol inhibited primary cloning of both D^+ and D^- variants of the leukemic cell lines while even high concentrations were not inhibitory to normal CFU-c; indeed in some studies significant enhancement of normal myelopoiesis was observed. 1,25-dihydroxyvitamin D_3 has proved effective in clinical trials for treatment of patients with postmenopausal osteoporosis [13], suggesting the feasibility of similar trials in patients with leukemia.

A combination of biological response modifiers such as endotoxin-induced GM-DF and vitamin analogues, either alone or as an adjunct to chemotherapy, offers a new approach to treatment of myeloid leukemias and myeloproliferative disorders currently refractory to conventional therapy.

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