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# All-*trans*-retinoic acid effects the growth, differentiation and apoptosis of normal human myeloid progenitors derived from purified CD34+ bone marrow cells

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**We have previously shown that all-*trans* retinoic acid (ATRA) increases the number of CFU-GM colonies grown from unseparated human bone marrow cells with crude sources of colony stimulating factors. In this study, we further characterized the effect of ATRA on the growth of CFU-GM stimulated by individual cytokines from multiple samples of CD34 enriched or purified human bone marrow cells. The number of IL-3- or GM-CSF-induced CFU-GM with 3 10 7 M ATRA was 3.25 ± 1.13, and 2.17 0.8-fold greater respectively, compared to controls without ATRA, while G-CSF had no effect and the ratio of colony-induced with or without ATRA was 1.06 0.17 (*P* 0.00012). No colonies grew with ATRA IL-6 or ATRA without a cytokine. Maximum enhancing effect on IL-3-induced CFU-GM occurred when ATRA was added on day 2, gradually diminished when delaying ATRA, and in cultures of day 9 or older adding ATRA had no effect. In 14 days liquid cultures of purified CD34 cells with IL-3, ATRA increased the number of myeloid differentiated cells to 91–95%, compared to 37–70% with IL-3 alone. In addition, the number of apoptotic cells using the annexin V method increased after 14 days from 5.1% with IL-3 to 17.1% with IL-3 ATRA and by the TUNEL *in situ* method from 10–26% to 60–95%, respectively. This study demonstrates that ATRA consistently enhances the growth of myeloid progenitors from CD34 cells. This effect is dependent on the stimulating cytokine, suggesting the myeloid cells responding to ATRA are the less mature CFU-GMs that are targets of IL-3 and GM-CSF and not the G-CSF-responding mature progenitors. The growth stimulation by ATRA and IL-3 is also associated with granulocyte differentiation and increased apoptosis. These studies further suggest a potential role of pharmacological doses of ATRA on the development of normal human hematopoietic cells.** *Leukemia* (2000) **14,** 874–881.
**Keywords:** retinoic acid; CD34+ cells; growth; differentiation; apoptosis

## Introduction

Vitamin A and its derivatives (retinoids) play an important role in embryogenesis as well as the growth, differentiation and regulation of various types of normal and malignant cells[1–4] including hematopoietic cells.[5–8] The most established effect of all-*trans* retinoic acid (ATRA) is its ability to induce differentiation of acute promyelocytic leukemia (APL) cells, both *in vitro*,[9,10] and *in vivo*[11,12] and is associated with complete hematological remission in almost all patients with APL when used in doses that provide supraphysiological serum concentrations.[13,14] Of importance, APL is associated with a balanced t(15;17) translocation that rearranges one allele of the gene encoding the retinoic acid receptor alpha (RAR ) on chromosome 17 with the promyelocytic (PML) gene on chromosome 15.[15–17] The translocation is unique for APL and has never been described in other cancers.[18]

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It is most likely that the differentiation of APL cells by ATRA is mediated through the APL-specific PML/RAR fusion transcript,[19–22] but this does not exclude the possibility that normal hematopoietic cells, which have two normal RAR alleles, would also respond to ATRA. Employing murine cells, Tsai and Collins[6] have shown that normal RAR receptor in synergy with GM-CSF, is essential for normal differentiation of myeloid progenitors. In these experiments, the differentiation of normal mouse bone marrow cells by GM-CSF could be blocked at the promyelocytic stage, by transducing the cells with a retroviral vector that harbored a truncated RAR gene (LRAR 403SN), exhibiting dominant negative activity against normal RAR . Further, supraphysiological concentrations of ATRA were shown to overcome this block, and together with GM-CSF, induce terminal cellular differentiation.[6] Using human bone marrow mononuclear cells, we reported several years ago that supraphysiological concentrations of ATRA influenced the growth *in vitro* of normal myeloid progenitor cells,[23] by increasing approximately twofold the number of CFU-GM colonies induced by crude preparations of colony-stimulating factor. This observation was later confirmed by several other groups, using unseparated,[24–27] more purified hematopoietic progenitors[28] or CD34+ cells.[29–31] In these reports, crude conditioned medium or a mixture of cytokines was used to stimulate CFU-GM colony growth.

Physiologically, low levels (nanomolar range) of ATRA are present in normal plasma. However, pharmacological doses of ATRA have been administered in several clinical trials treating different types of tumors, resulting in higher plasma ATRA levels. Our experiment focuses on the effect *in vitro* of supraphysiological concentrations (micromolar range) of ATRA, which are the levels attained in the plasma of patients treated with this drug. In our earlier experiments,[23] we grew CFU-GM colonies from a heterogeneous mixture of mononuclear bone marrow cells and used different conditioned media as sources of colony-stimulating factor. In the current study, we used a homogeneous population of CD34+ enriched or purified normal human bone marrow cells and examined the effect of ATRA on CFU-GM growth, stimulated by the individual hematopoietic cytokines IL-3, GM-CSF, G-CSF, or IL-6. The experiments were performed on multiple samples to investigate if the growth-stimulating effect of ATRA on myeloid progenitors is consistent in samples from different individuals. We also examined the effect of ATRA on IL-3-induced myeloid differentiation and apoptosis of CD34+ cells.

## Materials and methods

### *Cytokines and reagents*

The following recombinant cytokines were used: IL-3 (Genzyme, Cambridge, MA, USA), GM-CSF (Immunex, Seattle, WA, USA), G-CSF (Amgen, Thousand Oaks, CA, USA)

and IL-6 (Stem Cell Technologies, Vancouver, BC, Canada). ATRA (Catalog No. R-2625; Sigma, St Louis MO, USA) was dissolved in absolute ethanol at 1 mg/ml to create a stock solution that was kept at –20°C up to 5 days. Dilutions to the final concentrations were made with PBS. The maximum concentration of ethanol in the culture was 0.1% and did not influence the cellular growth or differentiation.

### *Cell purification and phenotype analysis*

Bone marrow cells free of tumor were obtained from harvests of patients undergoing autologous transplantation. Mononuclear cells were isolated by Ficoll–Hypaque (Organon; Teknika, Durham, NC, USA) gradient centrifugation. $CD34^+$ cells were first enriched with an immunoabsorption column (CellPro, Bothel, WA, USA) according to the manufacturer's instructions. Briefly, cells were incubated with biotinylated anti-CD34 antibodies for 25 min at room temperature. After washing, the cells were loaded on an avidin column and the $CD34^+$ cells that bound to the column were eluted by mechanical agitation and washed. The purity of the $CD34^+$ cells isolated by this method was only 55–96%. In order to obtain a higher degree of purification in certain experiments we used a second step of flow cytometric separation. Because of the requirement for large numbers of $CD34^+$ cells and the very long time required to isolate them by flow cytometry, the two-step purification was not performed in all the experiments. Thus, $CD34^+$ cells enriched by the immunoabsorption column were incubated with 20 l anti-CD34 MoAb (anti-HPCA2 epitope; Becton Dickinson, San Jose, CA, USA), conjugated with either FITC or PE at approximately $1 \times 10^6$ cells, for 30 min at 4°C in the dark, and then washed twice with PBS. The cells were then resuspended in RPMI-1640 and sorted with an appropriate gate to exclude cellular debris and aggregates. Flow cytometric separation was performed using EPICS-ELITE ESP Cell Sorter (Coulter, Hialeah, FL, USA) with data analysis performed by ELITE ESB workstation software. The purity of $CD34^+$ cells separated by the two steps was 95% (Figure 1).

### *CFU-GM colony formation*

Enriched or purified $CD34^+$ cells were placed at $1 \times 10^4$ cell/ml in pre-prepared 0.9% methylcellulose containing Iscove's medium, 30% FBS, 1% bovine serum albumin, $10^{-4}$ M -mercaptoethanol and 2 mM L-glutamine (catalog No. HCG4230, Stem Cell Technologies). The concentration of the cytokine added was IL-3 100 U/ml, GM-CSF 50 ng/ml, or G-CSF 75 ng/ml or IL-6 50 ng/ml, which were found to be the optimal concentrations (data not shown). The culture mixtures were placed in 35 mm dishes (Irvine Scientific, Irvine, CA, USA) for 14 days at 37°C in a humidified incubator containing 5% $CO_2$ in air and colonies ( 20 cells) were counted with an inverted microscope. Three dishes were plated for each experimental point. The CFU-GM score of each sample was obtained from the mean of the individual CFU-GMs in the triplicate dishes. When several samples were used for an experiment, the results were calculated as the mean ± s.d. of the triplicate means.

### *Analysis of differentiation*

Differentiation was examined only on cells purified by two steps of $CD34^+$ separation. The cells were placed at $10^5$ cell/ml in liquid medium (catalog No. 430-220, GIBCO, Gaithersburg, MD, USA), containing 10% FBS (HyClone, Logan, UT, USA) and IL-3 100 U/ml in the presence or absence of $3 \times 10^{-7}$ M ATRA. IL-3 was included in the culture to maintain a similar culture condition as the colony formation growth assay. After 7 and 14 days, the cells were washed and analyzed for myeloid differentiation by NBT reduction after mixing 25 000 cells in 250 l PBS with an equal volume of a solution containing 0.2% NBT (Sigma) and 100 ng/ml of 12-O-tetradecanoylphorbol 13-acetate (TPA) for 25 min at 37°C. The cells were resuspended in 20 l of PBS, air-dried slides were prepared by cytospin, and stained with Leukostat staining kit (catalog No. CS-430D; Fisher, Pittsburgh, PA, USA). NBT reduction was detected by formation of blue–black formazan deposits in the cells. At least 200 cells were counted.

In addition, 14 day CFU-GM colonies grown in methylcellulose with IL-3 (with or without ATRA), were picked with a micropipette and the cells from each individual culture dish were pooled in PBS. The cells were washed and incubated for 25 min at 37°C in media with equal volume of 0.2% of NBT dissolved in PBS containing 100 ng of freshly diluted TPA per ml. The percentage of cells containing intracellular blue–black formazan deposits obtained from each individual culture dish was determined on leukostat cytospin slides. Each

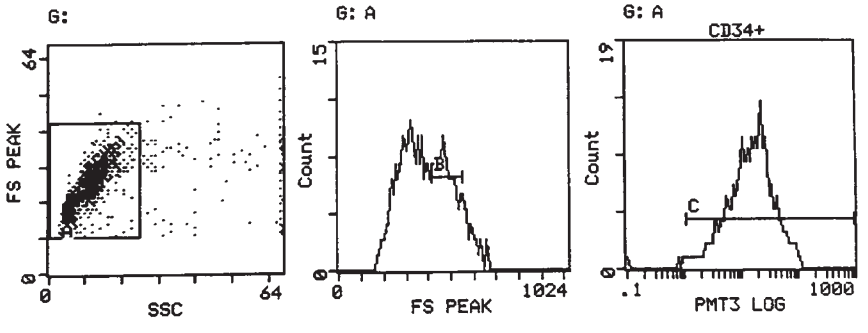


**Figure 1** Flow cytometric analysis of bone marrow cells enriched for $CD34^+$ cells: 96.7% $CD34^+$ cells after column avidin-biotin immunoabsorption followed by a second step of flow cytometric sorting with anti-CD34 antibodies (right panel); flow cytometry of the cells after the first separation step (middle panel); gated cells for flow cytometry (left panel).

experimental point was set in two dishes and the results expressed as the mean of the duplicate.

### *Apoptosis assay*

All apoptosis assays were performed on cells purified by the two steps of CD34 separation. The cells were placed at $10^5$ cell/ml in liquid medium containing 10% FBS and IL-3 100 U/ml in the presence or absence of $3 \times 10^{-7}$ ATRA and samples were taken on days 7 and 14 of culture for analysis. IL-3 was included in the culture since cells undergo apoptosis when growth factors are withdrawn[32,33] as well as to maintain similar culture condition to cell growth assay. Apoptosis was first assayed by flow cytometry binding of annexin V and propidium iodide (PI) using an Apoptosis Detection Kit (catalog No. KNX50, R & D Systems, Minneapolis, MN, USA).[34] During the early phase of apoptosis, before nuclear condensation had started, the cells exposed phosphatidylserine on the outer membrane which binds to annexin V. Cells with compromised membranes will allow PI to bind to cellular DNA. Live cells will not stain with either fluorochrome, necrotic cells will stain with both fluorochromes while cells undergoing apoptosis will stain only with annexin V. CD34+ cells were washed with cold PBS and $1 \times 10^5$ cells per 100 l of binding buffer and mixed with 10 l FITC-conjugated annexin V (10 g/ml) and 10 l of PI (50 g/ml) for 15 min at room temperature. Flow cytometric analysis was performed using a FACSCAN flow cytometer (Becton Dickinson) with analysis by Consort 30 software. A second assay to identify apoptosis in individual cells was performed by the TUNEL (TdT-mediated dUTP-nick end labeling) *in situ* labeling of DNA fragmentation in the nucleus, using an Oncor Apoptag plus kit (catalogue No. 57101-KIT; Oncor, Gaithersburg, MD, USA), as previously described.[35] Briefly, the CD34+ cells were cytocentrifugated on glass slides, air-dried, fixed with 4% neutral buffered formaldehyde and washed with PBS. Endogenous peroxidase was quenched by adding 3% hydrogen peroxide for 5 min. Terminal deoxynucleotidyl transferase (TdT) enzyme was added to catalyze binding of digoxigenin-nucleotide triphosphate to the 3 -OH ends at sites of DNA breaks. The slides were incubated with peroxidase-conjugated anti-digoxigenin and diaminobenzidene and counter stained with methylgreen. The high concentration of 3 -OH ends generated by DNA fragmentation are localized in morphological identifiable nuclei apoptotic bodies that are stained and can be visualized *in situ*.

## Results

### *Effect of ATRA on CFU-GM colony formation from CD34+ cells*

The concentration of ATRA used in the experiments was $3 \times 10^{-7}$ M which was the optimal concentration for stimulating CFU-GM from unseparated bone marrow cells in our earlier report.[23] The following experiments were performed with CD34+ enriched to 55–96% by the avidin-biotin immunoabsorption column.

Using the same eight bone marrow samples, each from a different donor, we found that ATRA significantly increased the number of CFU-GM colonies from all samples induced by IL-3 or GM-CSF compared to their respective control cultures without ATRA ($P = 0.0004$ and $P = 0.008$, respectively) (Table 1). In contrast, the number of G-CSF-induced CFU-GM colonies did not increase in the presence of ATRA (Table 1). In the eight samples, the increment in the colony count by ATRA was significantly higher for IL-3-induced CFU-GM (mean ± s.d.: 3.25 ± 1.13-fold; range 1.93–5.63-fold) than for GM-CSF (2.17 ± 0.8-fold; range 1.43–3.5-fold), or for G-CSF colonies (1.06 ± 0.17-fold; range 0.71–1.25-fold) (analysis of variance $P = 0.00012$). The mean (± s.d.) number of CFU-GM in samples obtained from a group of 20 patients (including 12 patient samples studied only with IL-3) was 78 ± 47 colonies per $10^4$ cells with IL-3 but without ATRA, and 214 ± 123 per $10^4$ cells with IL-3 plus ATRA; the mean increment was 3.13 ± 1.17-fold (range 1.39–5.76). Sixteen of 20 (80%) samples showed a two-fold or more CFU-GM increment. In addition, in the presence of ATRA, the IL-3-induced colonies appeared larger in size than those that developed without ATRA. IL-6 did not induce CFU-GM colony formation either with or without ATRA (Table 1). In separate experiments, we demonstrated that the concentration of $3 \times 10^{-7}$ M of ATRA was optimal for its effect on colony formation employing the different cytokines (data not shown).

In the absence of ATRA the baseline number of colonies grown with G-CSF was much higher than with IL3 or GM-CSF. It is therefore possible that ATRA did not increase the number of G-CSF-induced colonies because the growth capability of CFU-GM colonies was saturated. To test this possibility we set two experiments to measure the effect of ATRA on colonies stimulated by the combination of G-CSF and IL-3. In the presence of ATRA, the number of colonies stimulated by G-CSF increased when IL-3 was added, indicating that the lack of ATRA effect on G-CSF induced colonies was not because the cultures had reached their maximal growth capacity (Table 2).

ATRA alone in the absence of a cytokine in the culture, did not induce colonies from CD34+ enriched cells. In addition, only a very small number of IL-3-induced CFU-GM colonies grew from the column non-absorbed CD34− cells in the presence or absence of ATRA (1.0 ± 0.6 and 4.1 ± 3.8 CFU-GM/$10^4$ cells, respectively). This CD34− population contained a median of 1.3% (range 0.1–4.5%) CD34+ cells which were most likely responsible for the few colonies which were observed.

In the above experiments, CD34+ cells were exposed to ATRA from the beginning of the culture. In the next set of experiments, we determined how long the developing CFU-GM colonies remained sensitive to the growth enhancing effect of ATRA, by adding ATRA to the top of the methylcellulose dishes after a delay of 1–13 days from the initiation of the cultures. Figure 2 shows that the enhancing effect on IL-3- or GM-CSF-induced colonies was maximal when ATRA was added on the 2nd day of the culture, and then gradually decreased as the addition of ATRA was delayed. By adding ATRA to the cultures after more than 9–10 days, the number of colonies no longer increased compared to cultures without ATRA. ATRA had no significant effect on G-CSF-induced colonies when added at any time interval (Figure 2).

In order to determine if ATRA had a direct effect on CD34+ cells, we further enriched the column immunoabsorbed CD34+ cells to a purity of greater than 95% by a second purification step using flow cytometry sorting to remove almost all potential accessory non-CD34+ cells. Figure 3 shows a slight drop in colony growth in the more purified cells, but the degree of enhancement by ATRA was similar (mean ± s.d. 3.6 ± 1.1-fold) when compared to that obtained after the first step of CD34+ cell enrichment (3.2 ± 1.4-fold).

**Table 1** Effect of ATRA on number of CFU-GM induced by different cytokines

| Sample No. | IL-3 (100 U/ml) ATRA | | GM-CSF (50 ng/ml) ATRA | | G-CSF (75 ng/ml) ATRA | | IL-6 (50 ng/ml) ATRA | |
|---|---|---|---|---|---|---|---|---|
| | – | + | – | + | – | + | – | + |
| 1 | 21 | 76 | 111 | 159 | 106 | 75 | 0 | 0 |
| 2 | 27 | 152 | 207 | 356 | 161 | 191 | 3 | 2 |
| 3 | 72 | 210 | 155 | 290 | 123 | 130 | 2 | 5 |
| 4 | 163 | 314 | 479 | 586 | 423 | 384 | 10 | 10 |
| 5 | 64 | 132 | 250 | 718 | 481 | 527 | | |
| 6 | 14 | 58 | 32 | 112 | 73 | 77 | 0 | 0 |
| 7 | 73 | 203 | 433 | 726 | 311 | 371 | | |
| 8 | 97 | 284 | 230 | 701 | 314 | 393 | | |
| Mean ± s.d. | 66 ± 46 | 179 ± 86 | 237 ± 14 | 456 ± 240 | 249 ± 145 | 268 ± 160 | 3 ± 3.7 | 3.4 ± 3.8 |
| Mean ± ATRA | | 3.25 | | 2.17 | | 1.06 | | |

CD34+ cells were separated by the immunoabsorption column (first-step separation) and incubated with or without $3 \times 10^{-7}$ M ATRA and the respective cytokine. CFU-GM colonies were counted after 14 days in culture. The same eight patient samples were used. The results are expressed as number of colonies per $1 \times 10^4$ CD34+ cells. Each sample was plated in three dishes. The CFU-GM score of each sample was obtained from the mean of the individual CFU-GMs in the triplicate dishes. With several samples for each experimental point the results were calculated as the mean ± s.d. of the triplicate means of each sample.

± ATRA: the ratio between the number of colonies with and without ATRA calculated first for each sample and then the mean of the samples is recorded.

**Table 2** CFU-GM grown with G-CSF plus IL3 in comparison with each cytokine alone

| IL-3 | | G-CSF | | G-CSF + IL-3 | |
|---|---|---|---|---|---|
| –ATRA | +ATRA | –ATRA | +ATRA | –ATRA | +ATRA |
| 21 | 76 | 106 | 85 | 131 | 197 |
| 27 | 152 | 161 | 180 | 219 | 292 |

See footnote of Table 1 (two experiments).

### *Effect of ATRA on myeloid differentiation of CD34+ cells*

Using CD34+ cells purified to 95% after the two separation steps, we measured myeloid differentiation by the ability to reduce NBT by production of superoxide. After 7 days in liquid medium in the presence of IL-3, the number of NBT-positive cells was similar with or without ATRA. After 14 days, the number of NBT positive cells was significantly higher in the cultures containing ATRA (Table 3). To further evaluate differentiation, seven to 10 individual 14-day CFU-GM colonies were picked and pooled from each individual culture plate and stained with BMT. In two separate experiments with IL-3, 80% and 83% of the cells in the colonies grown with ATRA were NBT positive, compared to 43% and 58%, respectively, of the cells in the colonies without ATRA.

### *Effect of ATRA on apoptosis of CD34+ cells*

Employing CD34+ cells purified to 95% after the two separation steps, we measured apoptosis by two methods. Using binding of annexin V, 5.1% of the total cells cultured for 14 days with IL-3 were apoptotic, while the proportion of the apoptotic cells increased to 17.1% with addition of $3 \times 10^{-7}$ M of ATRA. Using the *in situ* method, the percentage of apoptotic cells after 14 days in cultures with IL-3 and without ATRA was relatively low but it markedly increased when ATRA was added to the culture (Table 4). Of note, higher percentages of apoptotic cells were detected in the IL-3 plus ATRA cultures by the *in situ* assay than by the annexin V method. A smaller increase in the percentage of apoptotic cells was also seen by the *in situ* method in day 7 cultures with IL-3 plus ATRA when compared to cultures with IL-3 alone (Table 4).

## Discussion

In this study, we further characterized the effect of pharmacological ATRA concentrations on normal human enriched and purified CD34+ hematopoietic progenitor cells. We limited the experiments to studying myeloid progenitors using only the CFU-GM assay. The results confirmed our previous observations[23] with unfractionated bone marrow cells, that ATRA, in the presence of colony-stimulating factors, increases *in vitro* the number of CFU-GM colonies. We also confirmed with CD34+ enriched cells, that ATRA itself does not act as a colony-stimulating factor, and does not induce colony formation in the absence of a cytokine. For example, using IL-6, which by itself does not stimulate colony growth, ATRA was unable to induce CFU-GM. Thus, myeloid progenitors probably must first be stimulated to grow by an appropriate cytokine before ATRA can stimulate further proliferation. The effect of ATRA is directly on CD34+ cells, and not mediated through accessory cells, since the stimulating effect was not reduced when the CD34+ purity was increased from an average of 79% to greater than 95%. Similarly, previous observations with unfractionated bone marrow cells demonstrated that removal of mononuclear cells did not change the enhanced colony growth with ATRA.[23,26]

Utilizing molecularly defined cytokines, we demonstrated that the enhancing effect of ATRA on myeloid progenitors is dependent on the type of cytokine employed. The greatest effect was seen with IL-3, slightly less enhancement with GM-CSF, but no enhancement by ATRA in colonies induced by G-CSF. Tohda *et al*,[36] using unfractionated bone marrow cells, also reported that ATRA stimulated CFU-GM induced by IL-3 or GM-CSF but inhibited colonies grown with G-CSF. In the

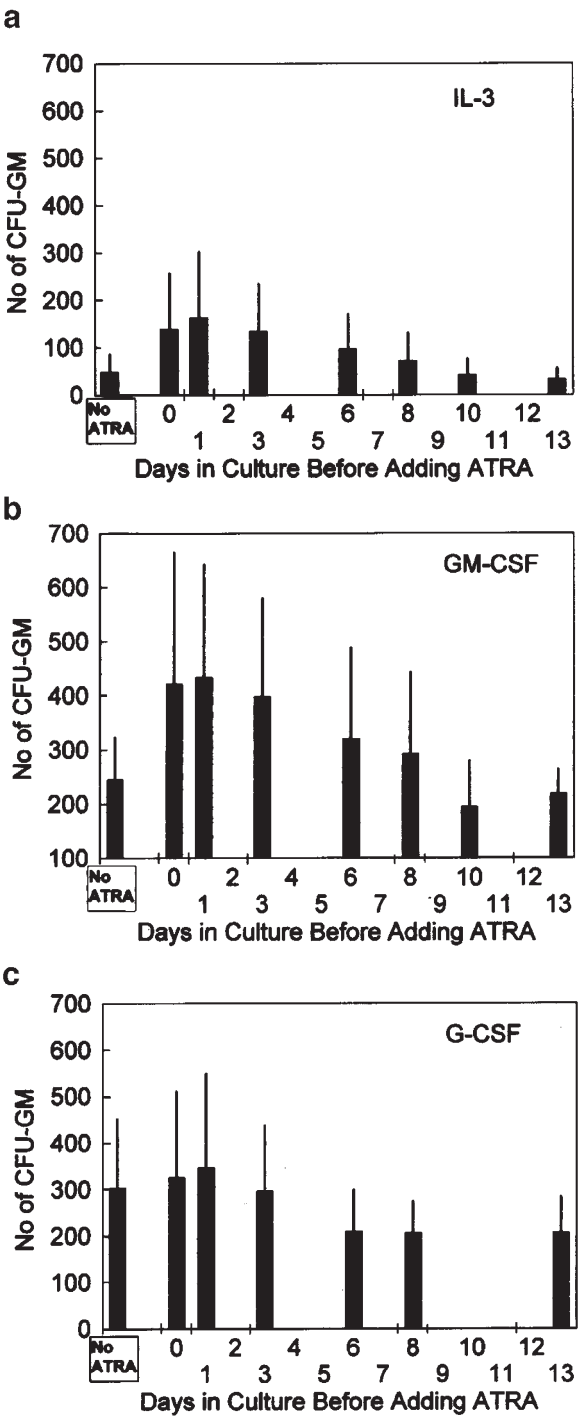


**Figure 2** CFU-GN colony grown with ATRA added at different time intervals. The cells were CD34+ separated by the two-step separation (see legend to Figure 1) and incubated at $1 \times 10^4$ cells per 1 ml with the respective cytokine (G-CSF, GM-CSF or IL-3) with $3 \times 10^{-7}$ M ATRA added to the top of petri dishes on days 1–13 after start of culture. CFU-GM colonies were counted after 14 days in culture. The results were expressed as the number of colonies grown per $1 \times 10^4$. Three to six samples were studied and the results are expressed as the mean ± s.d. percentage of controls. (a) 100 U/ml IL-3, (b) 50 ng/ml GM-CSF or (c) 75 ng/ml G-CSF.

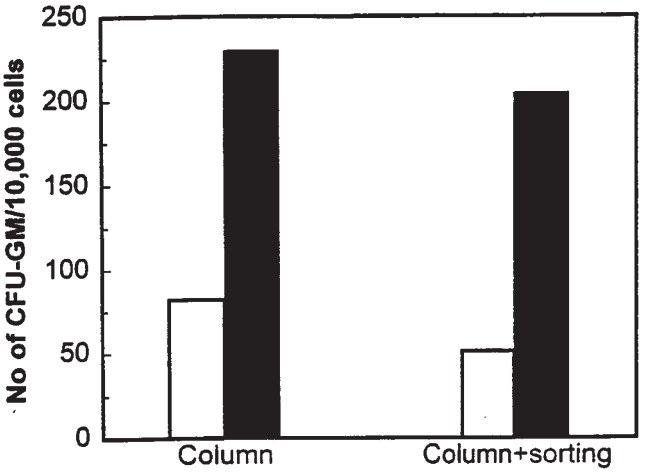


**Figure 3** ATRA enhancement of IL-3-induced CFU-GM after the first and second separation. The effect of ATRA on CD34+ enriched to 65–96.5% (mean ± s.d. 79 ± 9%) by first immunoabsorption column and of CD34+ enriched to 95% by a second step of flow cytometric sorting were similar. See flow cytometric analysis in Figure 1. Cells were incubated with 100 U/ml IL-3 and with $3 \times 10^{-7}$ M ATRA ( ) or without ATRA ( ). CFU-GM colonies were counted after 14 days. The results represent the mean of the number of colonies per $1 \times 10^4$ cells cultured from the same six patient samples.

only other study using CD34+ purified cells stimulated by individual cytokines reported by Smeland *et al*,[37] in which only two cell samples were employed, ATRA enhanced GM-CSF-induced CFU-GM colonies while it inhibited the growth of G-CSF-induced colonies; IL-3 was not examined. Taken together, the differential effect of the three individual cytokines, IL-3, GM-CSF, and G-CSF on ATRA modulation of myeloid progenitor growth appears similar in the three studies.[36,37] We were also able to show that this is a consistent phenomenon, verified on samples from multiple subjects. These results suggest a relationship between ATRA growth stimulation and the cytokine-responding target cells. IL-3 and GM-CSF stimulate the growth of overlapping but less mature CFU-GM, while G-CSF stimulates more differentiated myeloid progenitors.[38,39] The ATRA enhancing activity on CFU-GM grown with IL-3 and GM-CSF and lack of effect with G-CSF, probably implies that the target cells for ATRA growth stimulation are less mature IL-3 and GM-CSF-responding myeloid progenitors but not the more mature G-CSF-responding progenitor cells. Our time-delayed experiments support this possibility by showing that the enhancing activity of ATRA was maximal when the compound was added early in the course of the culture, while the colonies were still at their early stages of maturation. The response progressively declined as the addition of ATRA was delayed, and was no longer observed after the colonies had passed a certain degree of development. Thus, the growth enhancing activity of pharmacological levels of ATRA appears to be limited to a relatively early stage of the myeloid progenitor maturation and disappears as the cells differentiate.

The mechanism whereby ATRA increases the number of normal CFU-GM in culture is not clear. It is unlikely that newly formed cells within the developing colonies would serve as accessory cells providing additional growth factors, since our time-delayed experiments showed that the effect of ATRA diminishes as the colony increases in size. It is possible that ATRA stimulates the recruitment of additional preformed myeloid progenitors that otherwise die, to form colonies by

**Table 3** Effect of ATRA on IL-3-induced differentiation of CD34+ cells

| | Day | –ATRA | | | +ATRA | | |
|---|---|---|---|---|---|---|---|
| | | NBT+ (%) | Cell number ($10^{-3}$) | | NBT+ (%) | Cell number ($10^{-3}$) | |
| | | | Total | NBT+ | | Total | NBT+ |
| | 0 | 0 | 100 | 0 | 0 | 100 | 0 |
| Sample 1 | 7 | 33 | | | 34 | | |
| | 14 | 70 | 308 | 215 | 95 | 416 | 395 |
| Sample 2 | 7 | 39 | | | 40 | | |
| | 14 | 37 | 185 | 69 | 91 | 180 | 163 |

100000 CD34+ cells that were separated by immunoabsorption column and flow cytometry sorting (two steps) and incubated for 7 and 14 days in 2 ml liquid medium with 100 U/ml IL-3 and with or without $3 \times 10^{-7}$ M ATRA. The results represent the percentage of NBT-positive cells, the total number of viable cells and total number of viable NBT + cells (two experiments).

**Table 4** Effect of ATRA on IL-3-induced apoptosis of CD34+ cells (*in situ* method)

| | Sample No. 1 | | Sample No. 2 | |
|---|---|---|---|---|
| | Day 7 | Day 14 | Day 7 | Day 14 |
| –ATRA | 4% | 10% | 5% | 26% |
| +ATRA | 39% | 95% | 40% | 69% |

CD34+ cells that were separated by immunoabsorption followed by flow cytometry sorting (two steps) were incubated in liquid medium with 100 U/ml IL-3 and with or without $3 \times 10^{-7}$ M ATRA. The results represent the percentage of cells staining positive with peroxidase-conjugated anti-digoxigenin demonstrating *in situ* apoptosis after 7 and 14 days in culture (two experiments).

increasing their sensitivity to IL-3 and GM-CSF. An alternative possibility that ATRA shifts less mature multi-lineage progenitors towards a myeloid program of differentiation was not studied, and would require assays of multipotent progenitors. However, Tocci *et al*[30] showed with a mixture of cytokines and erythropoietin that ATRA shifts the differentiation of human fetal liver CD34+ cells from an erythroid/monocytic pathway towards the granulocytic lineage.

All-*trans* retinoic acid also intervenes in the programmed cell death and differentiation of CD34+. In our experiments, IL-3 prevention of apoptosis[32,33] was reversed and CD34+ cells died by programmed cell death, when ATRA was added to the cultures with IL-3. Similarly, Josefsen *et al*[40] described that ATRA opposed the anti-apototic activities of GM-CSF and G-GSC on CD34+. A dual effect of growth stimulation and apoptosis as seen with IL-3 and GM-CSF on CD34+, has also been described in the development of other organs.[41,42] In addition, our study showed that normal human CD34+ cells differentiate by ATRA and IL-3 to granulocytes capable of reducing NBT. Although not directly studied, it is possible that the growth and differentiation effect of ATRA are coupled as suggested for hematopoietic cells by several investigators.[43,44] Our experiments also show in CD34+ cells incubated with IL-3, that ATRA induces granulocytic differentiation in parallel with programmed cell death, a phenomenon which has been observed in other cell systems. For example, in the p19 mouse embryonal carcinoma cell line, ATRA induces differentiation toward neuronal cells in association with cellular apoptosis.[45,46] Also, the myeloid leukemia HL-60 cell line differentiates with ATRA to granulocyte-like cells[5] together with cellular apoptosis.[47,48]

Our *in vitro* experiments were limited to pharmacological concentrations of ATRA in the presence of individual cytokines and further confirm that this drug has biological activities on hematopoietic cells of the myeloid-lineage that have normal nuclear retinoid receptors. The *in vivo* correlates of the culture results are not clear. In general, clinical trials studying ATRA in cancer have not reported changes in neutrophil counts[49] except for leukocytosis in anecdotal cases[50] and in more than half of active acute promyelocyic leukemia (APL) cases.[51] It is possible, that in bone marrow of non-APL cancer patients an *in vivo* stimulatory effect of ATRA on the small numbers of normal myeloid progenitors is counterbalanced by other opposing mechanisms and does not become clinically apparent. In contrast, APL patients with active disease often exhibited a dramatic hyperleukocytosis while being treated with ATRA.[51] The rise in circulating leukemic cells is seen early in the course of treatment and returns to baseline with continuation of ATRA administration and further differentiation of the leukemic cells. Interestingly, APL patients in complete remission, that have few or no leukemic cells, do not exhibit leukocytosis while receiving ATRA as maintenance treatment.[13] In active APL, the bone marrow is essentially replaced by a homogenous population of malignant cells and it is possible that a potential *in vivo* effect of ATRA on their growth can be clinically expressed. We are speculating that the clinical hyperleukocytosis observed in APL patients during early ATRA administration, is reminiscent of the *in vitro* activity of ATRA on normal myeloid progenitors; growth enhancement of immature APL cells that subsequently undergo apoptosis with further differentiation.

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## References

1 Hoffman C, Eichele G. Retinoids in development. In: Sporn MB, Roberts AB, Goodman DS (eds). *The Retinoids: Biology, Chemistry and Medicine*, 2nd edn. Raven Press: New York, 1994, pp 387–442.
2 Smith MA, Parkinson DR, Cheson B, Friedman MA. Retinoids in cancer therapy. *J Clin Oncol* 1992; **10**: 839–864.
3 Means AL, Gudas LJ. The role of retinoids in vertebrate development. *Annu Rev Biochem* 1995; **64**: 201–233.
4 Goodman DW. Vitamin A and retinoids in health and disease. *New Engl J Med* 1984; **310**: 1023–1034.
5 Breitman TR, Selonik SE, Collins SJ. Induction of differentiation of human progranulocytic leukemia cell line (HL-60) by retinoic acid. *Proc Natl Acad Sci USA* 1980; **77**: 2936–2940.
6 Tsai S, Collins SJ. A dominant negative retinoic acid receptor blocks neutrophil differentiation at the promyelocytic stage. *Proc Natl Acad Sci USA* 1993; **90**: 7153–7157.
7 de The H, Marchio A, Tiollais P, Dejean A. Differential expression and ligand regulation of the retinoic acid receptor alpha and beta genes. *EMBO J* 1989; **8**: 429–443.
8 Largman C, Detmer K, Corral JC, Hack FM, Lawrence HJ. Expression of retinoic acid receptor alpha mRNA in human leukemia cells. *Blood* 1989; **74**: 99–102.
9 Breitman TR, Collins SJ, Keene BR. Terminal differentiation of human promyelocytic leukemic cells in primary culture in response to retinoic acid. *Blood* 1981; **57**: 1000–1004.
10 Chomienne C, Ballerini P, Balitrand N, Daniel MT, Fenaux P, Castaigne, Degos L. All *trans* retinoic acid in acute promyelocytic leukemia. II. *In vitro* studies: structure–function relationship. *Blood* 1990; **76**: 1710–1717.
11 Warrel RP Jr, de The H, Want Z-Y, Degos L. Acute promyelocytic leukemia. *New Engl J Med* 1993; **329**: 177–189.
12 Elliott S, Taylor K, White S, Rodwell R, Marlton P, Meagher D, Wiley J, Taylor D, Wright S, Timms P. Proof of differentiative mode of action of all-*trans* retinoic acid in acute promyelocytic leukemia using X-linked clonal analysis. *Blood* 1992; **79**: 1916–1919.
13 Tallman MS, Andersen JW, Schiffer CA, Appelbaum FR, Fausner JH, Ogden A, Sheperd L, Bloomfiled CJ, Head DR, Weinstein HJ, Woods WG, Rowe JM, Weirnick PA. A prospective randomized trial of all-*trans*-retinoic acid in induction and maintenance therapy for patients with acute promyelocytic leukemia. *New Engl J Med* 1997; **337**: 1021–1028.
14 Fenaux P, Le Delay MC, Castaigne S, Archibaud E, Chemoienne C, Link H, Guerci A, Duarte M, Daniel MT, Bowen D, Huebner G, Bauters F, Fegueux DN, Fey M, Sanz B, Lowenberg B, Maloisel F, Auzanneau G, Sadoum A, Gardia C, Bastion Y, Gasner A, Jacky E, Dombert H, Chastang C, Degos L, and the European APL 91 Group. Effect of all-*trans* retinoic acid in newly diagnosed acute promyelocytic leukemia. Results of a multicenter randomized trial. *Blood* 1993; **82**: 3241–3249.
15 de The H, Chomienne H, Lannotte M, Degos L, Dejean A. The t(15;17) translocation of acute promyelocytic leukemia fuses the retinoic acid receptor gene to a novel transcribed locus. *Nature* 1990; **347**: 358–361.
16 Kakizuka A, Miller WH, Umesono K, Warrell RP, Frankel SR, Murty VVVS, Dmitrovsky E, Evans RM. Chromosomal translocation t(15;17) of human acute promyelocytic leukemia fuses RAR alpha gene with a novel putative transcription factor, PML. *Cell* 1991; **66**: 663–674.
17 Pandolfi PP, Grignani F, Alcalay M, Mencarelli A, Biondi A, Lo Coco F, Grignani F, Pelicci PG. Structure and origin of the acute promyelocytic leukemia myl/RAR- cDNA and characterization of its retinoid-binding and transactivation properties. *Oncogene* 1991; **6**: 1285–1292.
18 Grignani F, Fagioli M, Alcalay M, Longo L, Pandolfi PP, Donti E, Biondi A, LoCoco F, Grignani F, Pelicci PG. Acute promyelocytic leukemia: from genetics to treatment. *Blood* 1994; **83**: 10–25.
19 Grignani FR, Ferruci PF, Testa U, Talamo G, Fagioli M, Alcalay M, Mencarelli A, Grignani F, Peschle C, Nicoletti I, Pelicci PG. The acute promyelocytic leukemia specific PML/RAR fusion protein inhibits differentiation and promotes survival of myeloid precursor cells. *Cell* 1993; **74**: 423–431.
20 Mernick A, Licht JD. Deconstructing a disease: RAR , its fusion partners and their roles in the pathogenesis of acute promyelocytic leukemia. *Blood* 1999; **93**: 3167–3215.
21 Dermine S, Grignani F, Clerici M, Nervi C, Sozi G, Talamo GP, Marchesi E, Formelli F, Parmiani G, Pelicci PG, Gambacorti-Passerini C. Occurrence of resistance to retinoic acid in the acute promyelocytic leukemia cell line NB4 is associated with altered expression of the PML/RAR protein. *Blood* 1993; **82**: 1573–1577.
22 Degos L, Dombret H, Chomienne C, Daniel MT, Miclea JM, Chastang C, Castaigne S, Fenaux P. All-*trans* retinoic acid as a differentiating agent in the treatment of acute promyelocytic leukemia. *Blood* 1995; **85**: 2643–2653.
23 Douer D, Koeffler HP. Retinoic acid enhances colony stimulating factor-induced clonal growth of normal human myeloid progenitor cells *in vitro*. *Exp Cell Res* 1982; **138**: 193–198.
24 Nagler A, Riklis I, Kletter Y, Tatarsky I, Fabian I. Effect of 1, 2-dihydroxy vitamin D3 and retinoic acid on normal human pluripotent (CFU-mix) erythroid (BFU-E) and myeloid (CFU-C) progenitor cell growth and differentiation patterns. *Exp Hematol* 1986; **14**: 60–65.
25 Aglietta M, Piacibello W, Stacchini A, Sanavio F, Gavosto F. *In vitro* effect of retinoic acid on normal and chronic myeloid leukemia granulopoiesis. *Leukemia Res* 1985; **9**: 879–885.
26 Gratas C, Menot ML, Dresch C, Chomienne C. Retinoic acid supports granulocytic but not erythroid differentiation of myeloid progenitors in normal bone marrow cells. *Leukemia* 1993; **7**: 1156–1162.
27 Tobler A, Dawson MI, Koeffler HP. Retinoids: structure–function relationship in normal and leukemic hematopoiesis *in vitro*. *J Clin Invest* 1986; **78**: 303–309.
28 Labbaye C, Voltier M, Testa U Gaimpaolo A, Meccia E, Sterpetti P, Parolini I, Pelosi E, Bulgarini D, Cayre YE, Peschle C. Retinoic acid down modulates erythroid differentiation and GATA-1 expression in purified adult progenitor cultures. *Blood* 1994; **83**: 651–656.
29 Zauli G, Visani G, Vitale M, Gibellini D, Bertolaso L, Capitani S. All-*trans* retinoic acid shows multiple effects on the survival proliferation and differentiation on human fetal CD34+ hematopoietic progenitor cells. *Br J Haematol* 1995; **90**: 274–282.
30 Tocci A, Parolini I, Gabbianelli M, Testa U, Luchetti L, Samoggia P, Masella B, Russo G, Valtieri M, Peschle C. Dual action of retinoic acid on human embryonic/fetal hematopoiesis: blockade of primitive progenitor proliferation and shift from multipotent/erythroid/monocytic to granulocytic differentiation program. *Blood* 1996; **88**: 2878–2888.
31 van Bockstaele DR, Lenjou M, Snoeck HW, Lardon F, Stryckmans P, Peetermans ME. Direct effects of 13-*cis* and all-*trans* retinoic acid on normal bone marrow (BM) progenitors: comparative study on BM mononuclear cells and on isolated CD34+ BM cells. *Ann Hematol* 1993; **66**: 61–66.
32 Ormerod MG, Collins MKL, Rodriguez-Tarduchy G, Robertson D. Apoptosis in interleukin-3-dependent hematopoietic cells. Quantification by two flow cytometric methods. *J Immunol Meth* 1992; **153**: 57–67.
33 Sach L. The control of hematopoiesis and leukemia from basic biology to the clinic. *Proc Natl Acad Sci USA* 1996; **93**: 4742–4751.
34 Koopman G, Reutelingsperger CPM, Kuitjen GAM, Keehnen RMJ, Pals ST, van Oers MHJ. Annexin V for flow cytometric detection of phosphatidylserine expression of B cells undergoing apoptosis. *Blood* 1994; **84**: 1415–1422.
35 Gorczyca W, Gong J, Darzynkiewiczj A. Detection of DNA stand breaks in individual apoptotic cells by the *in situ* terminal deoxynucleatidyl transferase and nick translation assay. *Cancer Res* 1993; **53**: 1945–1953.
36 Tohda S, Minden MD, McCullock EA. Interaction between retinoic acid and colony stimulation factor affecting the blast cells of acute myeloblastic leukemia. *Leukemia* 1991; **5**: 951–957.
37 Smeland EB, Rusten L, Jacobsen SEW, Skrede B, Blomhoff R, Wang MY, Funderud S, Kvalheim G, Blomhoff HK. All-*trans* retinoic acid directly inhibits granulocyte colony-stimulating factor-

induced proliferation of CD34+ human hematopoietic progenitor cells. *Blood* 1994; **84**: 2940–2945.
38 Emerson SG, Yang YC, Clark SC, Long MW. Human recombinant granulocyte–macrophage colony stimulating factor and interleukin 3 have overlapping but distinct hematopoietic activities. *J Clin Invest* 1988; **82**: 1282–1287.
39 Caux C, Saeland S, Favre C, Duvert V, Mannoni P, Banchereau J. Tumor necrosis factor-alpha strongly potentiates interleukin-3 and granulocyte–macrophage colony-stimulating factor-induced proliferation of human CD34+ hematopoietic progenitor cells. *Blood* 1990; **75**: 2292–2298.
40 Josefsen D, Blomhoff HK, Lomo J, Blystad AK, Smeland EB. Retinoic acid induces apoptosis of human CD34(+) hematopoietic progenitors: involvement of retinoic acid receptors and retinoid X receptors depends on lineage commitment of the hematopoietic progenitor cells. *Exp Hematol* 1999; **27**: 642–653.
41 Ninomiya Y, Adams R, Morris-Kay GM, Eto K. Apoptotic cell death in neuronal differentiation of P19 EC cells: cell death follows reentry into S phase. *J Cell Physiol* 1997; **175**: 25–35.
42 Evans G, Harrington E, Fanidi A, Land H, Amati B, Bennett M. Integrated control of cell proliferation and cell death by the c-myc oncogene. *Phil Trans Royal Soc Long B Biol Sci* 1994; **345**: 269–275.
43 Lotem J, Sachs L. Coupling of growth and differentiation in normal myeloid precursors and the breakdown of this coupling in leukemia. *Int J Cancer* 1983; **32**: 127–134.
44 Metcalf D. The molecular control of cell division, differentiation commitment and maturation in hematopoietic cells. *Nature* 1989; **339**: 27–35.
45 Lee EY, Hu N, Yuan SS, Cox LA, Bradley A, Lee WH, Herrup K. Dual role of the retinoblastoma protein in cell cycle regulation and neuron differentiation. *Gene Dev* 1994; **8**: 2008–2021.
46 Okazawa H, Shimizu J, Imafuku I, Hamada H. Bcl-2 inhibits retinoic acid-induced apoptosis during the neural differentiation of embryonal stem cells. *J Cell Biol* 1996; **132**: 955–968.
47 Martin SJ, Bradley JG, Cotter TG. HL-60 cells induced to differentiate towards neutrophils subsequently die via apoptosis. *Clin Exp Immunol* 1990; **79**: 448–453.
48 Park JR, Robertson K, Hickstein DD, Tsai S, Hockenbery DM, Collins SJ. Dysregulated bcl-2 expression inhibits apoptosis but not differentiation of retinoic acid-induced HL-60 granulocytes. *Blood* 1994; **84**: 440–445.
49 Lee JS, Newman RA, Lippman SM, Hubner MH, Minor T, Raber MN, Krakoff IH, Hong WK. Phase I evaluation of all-*trans* retinoic acid in adults with solid tumors. *J Clin Oncol* 1993; **11**: 959–966.
50 Kahn MC, Luginbuhl W, Gaines L, Bratschi J. Leukocytosis associated with all-*trans*-retinoic acid therapy in metastatic non-small-cell lung cancer. *J Natl Cancer Inst* 1992; **84**: 1669–1670.
51 Frankel SR, Earldley A, Lauwers G, Weiss M, Warrel R. The 'retinoic acid syndrome' in acute promyelocytic leukemia. *Ann Intern Med* 1992; **117**: 292–296.