ACUTE PROMYEOLOCYTIC leukemia (APL) has aroused interest well beyond the hematologic field during the last 4 to 5 years. Two features, both of which are unique to APL, have attracted the attention of various sectors of biomedical research: (1) the remission of the disease obtained with retinoic acid (RA) treatment, whose mechanism of action consists in inducing the APL blasts to differentiate terminally; and (2) the presence in the APL blasts of an anomalous protein, the PML/RARα protein, a mutant of one of the retinoic acid receptors.

The high sensitivity of the promyelocytic blasts to RA makes APL a unique model for differentiation therapy. Because this antineoplastic strategy is radically different from conventional cytotoxic chemotherapy, it potentially provides a further tactic for controlling neoplastic growth. Several attempts were made to treat hematologic neoplastic disorders with differentiation therapy in the past. They were based on the observation that the differentiation block is a striking feature of the leukemic phenotype and that it can be reversed in vitro by a number of substances (eg, low-dose Ara-C). Unfortunately, on the whole, these clinical trials led to disappointing results. In contrast, differentiation therapy with RA induces clinical remission in the great majority of APL patients.

The PML/RARα fusion protein is formed as the consequence of a chromosomal translocation, t(15;17), that involves the PML and RARα genes. The anomalous protein is thought to play a crucial role in promyelocytic leukemogenesis. RARα is a member of the super-family of nuclear hormone receptors that are involved in fundamental biologic processes such as development and differentiation.

The discovery of a putative oncogenic RARα mutant, PML/RARα, raises questions of great importance concerning the normal function and oncogenic potential of all members of the nuclear receptor super-family.

The association of these two unique features of APL is paradoxical. On one hand, the alteration in the RA signaling pathway by PML/RARα could contribute to the leukemic phenotype, particularly the differentiation block and/or the outgrowth of the APL blasts. On the other hand, activation of the same signaling pathway by RA causes the disappearance of the leukemic blasts by inducing terminal differentiation. The story is even more intriguing when one considers that the differentiation block is the most distinctive component of the APL phenotype, as demonstrated by the modest proliferative rate of the promyelocytic blasts and the correlation between RA-induced differentiation and disease remission. Although the puzzle remains unresolved, should the PML/RARα protein, as expected, turn out to be responsible for both the differentiation block and sensitivity to RA, APL will be the first example of a neoplastic disease that can be treated by specifically targeting therapy to the transforming protein.

This review will summarize available information on the genetics of APL by trying to group what is known according to the three main questions that require an answer: (1) What is the role of PML/RARα in promyelocytic leukemia? (2) What is the mechanism of action of the PML/RARα protein? (3) What is the molecular basis of RA treatment? The aim is to provide the hematologist with the background information required to evaluate the numerous findings that will emerge in the near future. The more directly clinical implications of such studies will be also discussed.

THE 15;17 TRANSLOCATION

APL is characterized by a reciprocal translocation that involves chromosomes 15 and 17. As a consequence of this translocation two recombinant chromosomes are formed: 15q+ and 17q−. The chromosome breakpoints have been variously mapped to 15q22-q24 and 17q11-q21. Because the quality of the metaphases obtained from neoplastic promyelocytes is often poor, the reported breakpoint heterogeneity may reflect technical difficulties rather than molecular heterogeneity of the translocation. By combining chromosome banding and in situ hybridization techniques, our group mapped the translocation breakpoints to 15q24 and 17q21.

The chromosome break sites were isolated by four groups using distinct experimental approaches. The chromosome 15 breakpoint falls within a previously unknown gene, which was originally designated myl, but then renamed PML, for promyelocytes. The chromosome 17 breakpoint is located in the locus that encodes the retinoic acid receptor α (RARα). Two fusion genes are formed as a consequence of the translocation: the PML/RARα gene on the recombinant 15q+ chromosome and its reciprocal RARα/PML on the recombinant 17q− chromosome (Fig 1).

Expression of the t(15;17) is strictly limited to neoplastic promyelocytes; it has never been reported in any other neoplasia. There have been descriptions of chronic myeloid leukemia in blast crisis with a t(15;17) that, however, displayed the promyelocytic phenotype and rearrangements in PML and RARα.
The percentage of cases with the t(15;17) varies from 70% to 90%. It would be of great importance if the remaining 10% to 30% had, indeed, a normal karyotype, because it would imply heterogeneity in the genetic lesions underlying the disease. However, molecular analysis has shown rearrangements of PML and RARα genes in all cases with an apparently normal karyotype examined thus far. We recently analyzed two APL cases with apparently normal karyotypes using the fluorescence in situ hybridization technique (FISH) and found a submicroscopic translocation of PML on chromosome 17 in one and of RARα on 15 in the other (our unpublished results in collaboration with M. Lafage, Institut Paoli-Calmettes, Marseille, France). In summary, combined cytogenetic and molecular findings strongly suggest that t(15;17) is present in 100% APL cases.

The t(15;17) is often (70% to 90%) the only chromosomal anomaly seen in the neoplastic metaphases. However, additional karyotypic changes may accompany the t(15;17) and, as in other types of myeloid leukemia, trisomy 8 is the most common and our unpublished results, February, 1993).

Variant translocations are very rare in APL and usually involve 17 with chromosomes other than 15. Breakpoints in a 11;17 chromosome translocation have recently been cloned from a case of APL. The chromosome 17 breakpoint has been shown to lie within the RARα gene, while in chromosome 11 it is located in a previously unknown gene named PLZF (promyelocytic leukemia zinc finger), which has certain structural similarities with PML.

To conclude, the t(15;17) is associated exclusively with a promyelocytic phenotype and there are no other known chromosome alterations able to express this phenotype (aside from the t(15;17)), which suggests that the t(15;17) is implicated in the pathogenesis of APL. Furthermore, it would seem that of the two chromosomes, 17 is the only one indispensable for expression of the promyelocytic phenotype. However, this does not necessarily imply that only alterations in RARα are crucial in the pathogenesis of APL, because concomitant alterations in a second gene, almost always PML, occasionally PLZF, are invariably present. Therefore, it would seem that PML, PLZF, and other putative translocation partners of RARα must share common properties necessary for the pathogenesis of the disease.

PML/RARα AND ITS EFFECTS ON DIFFERENTIATION AND GROWTH OF HEMATOPOIETIC PRECURSORS

The centromere-telomere transcriptional orientation of both the PML and RARα loci is 5'-3'. As a result, in the PML/RARα chimeric gene, the PML and RARα portions are fused in a head-to-tail configuration and are under the transcriptional control of the PML promoter (Fig 1).

The chimeric PML/RARα gene is transcriptionally active in all cases of APL. The fusion transcript can be identified by Northern blotting using either PML or RARα cDNA probes which both hybridize to transcripts of 4.4 and 3.6 kb in approximately half of APL cases, and 4.0 and 3.2 kb in the remaining cases. The variable position of the chromosome 15 breakpoints (see below) and the alternative usage of two RARα polyadenylation sites are responsible for the different sizes of the PML/RARα transcripts. The PML/RARα fusion transcript has the potential to encode a PML/RARα fusion protein.

Because the expression of the PML/RARα protein and the emergence of the APL phenotype coincide, the working hypothesis is that PML/RARα is responsible for the transformed phenotype in APL; however, direct proof is still lacking (eg, ectopic expression of the fusion protein into normal hematopoietic precursors). Indirect evidence has, instead, been obtained from expressing PML/RARα in hematopoietic precursor cell lines. Results have provided evidence of biologic activities of PML/RARα that recapitulate critical features of the promyelocytic leukemia phenotype.

Effects on differentiation. Hematopoietic precursor cell lines can be driven to terminal differentiation by various inducers. For example, vitamin D3 (VD) or combined VD and transforming growth factor β1 (TGFβ1) treatment induces terminal monocytic differentiation of the U937 pro-monocytic cell line. U937 cells that express the PML/RARα protein fail to terminally differentiate, as shown by both surface marker analysis and functional tests (proliferation, phagocytosis). The effect of PML/RARα protein expression is dose dependent: differentiation is effectively blocked in U937 cells only when the PML/RARα expression levels are higher than those of the normal RARα protein, a situation similar to that found in the APL blasts. The same inhibitory activity of PML/RARα is observed when PML/RARα-expressing cells are exposed to low RA concentrations, comparable with the physiologic levels. Whereas the control cells undergo limited differentiation, the hematopoietic precursor cells containing PML/RARα do not differentiate at all.

The situation is completely reversed when the cells expressing the fusion protein are exposed to high RA concentrations, comparable with the peak levels achieved in the bloodstream of APL patients treated with RA. The percentage of cells that enter the differentiation program is higher in the PML/RARα-expressing cell population than in control cells. Therefore, a high concentration of RA converts the activity exerted by the fusion protein on differentiation from inhibitory to stimulatory.

Overall these results show that the PML/RARα fusion protein could alone account for two major features of the APL phenotype: the block of differentiation and the high sensitivity to RA.

At what level of the cascade of events associated with the differentiation process does PML/RARα exert its activity? In traditional models, stem cells undergo commitment processes that limit their self-renewal and direct their differentiation potential. Maturation begins as the cells exhaust their capacity to replicate. Hematopoietic precursor cell lines make good in vitro models for studying differentiation. Their potential to grow indefinitely reflects their capacity for self-renewal. When they are treated with various inducers, precursor cell lines proceed to commitment, lose their capacity for self-renewal, and become mature (Fig 2). In this setting, PML/RARα could interfere with the differentiation process during either the commitment or maturation processes. PML/RARα-expressing U937 cells treated with VD not only lose their capacity to differentiate but, to a large extent, also their self-renewal properties (our unpublished
Fig 1. Cytogenetic and molecular architecture of APL t(15;17). (A) FISH using a chromosome 17 painting probe on a metaphase from an APL patient. The larger hybridization spot identifies the residual normal chromosome 17, smaller spots the chromosome markers 15q+ and 17q-. (B) The normal chromosome 15 (#15) and 17 (#17) as well as the APL derivative chromosomes 15q+ (#15q+) and 17q- (#17q-) are shown diagramatically. The PML and RARα loci and the fusion genes PML/RARα on 15q+ and RARα/PML on 17q- are indicated.
results, April 1993). This suggests that PML/RARα acts by blocking maturation (Fig 2). Instead, in the presence of RA, the percentage of cells that enter the differentiation process without fully differentiating is higher than that of control cells, suggesting that PML/RARα acts by increasing the frequency of commitment events (our unpublished results) (Fig 2). Therefore, PML/RARα seems to exert dual functions on differentiation, one inhibitory and RA-independent, the other stimulatory and RA-dependent. The two activities are not apparently antagonistic because simultaneous addition of VD and RA induces terminal differentiation in PML/RARα-expressing cells (our unpublished results, August 1993).

Effects on cell growth. U937 cells expressing the PML/RARα protein do not undergo programmed cell death in conditions that induce apoptosis in control cells, like growth factor deprivation. Therefore, it seems that the PML/RARα protein promotes cell survival by inhibiting endogenously programmed death. Cell-cycle analysis of control and PML/RARα-expressing cells showed no significant variations of proliferation indices. Similarly, in the granulocyte-macrophage colony-stimulating factor (GM-CSF)-dependent cell line TF-1, survival after withdrawal of the growth factor is prolonged by the expression of PML/RARα (our unpublished results, March 1993).

In vivo labeling experiments have shown that APL blasts have a lower proliferation rate than blasts from other types of leukemias. By prolonging cell survival, PML/RARα could sustain the expansion of a population of leukemic cells with a low proliferative index. This effect, combined with the differentiation block, provides a cellular mechanism to account for the oncogenic potential of PML/RARα (Fig 2).

STRUCTURE AND MECHANISMS OF ACTION OF THE PML/RARα FUSION PROTEIN

On the basis of its structure and preliminary biochemical data it appears that PML/RARα may act as a multifunctional protein with the potential to interfere with the endogenous activation pathways of PML, RARα, and other members of the nuclear receptor family, such as thyroid hormone receptors (TR) and VD receptors (VDR).

A first step in understanding the mechanism(s) of action of PML/RARα is to define which RARα regions are retained in the fusion protein and to establish whether their corresponding functions are maintained or modified when they fuse with PML (Fig 3).

Wild-Type RARα Protein

RARα is a member of a retinoic acid receptor family that also includes RARβ and RARγ. A separate retinoid receptor family, the X receptors, includes RXRα, RXRβ, and RXRγ.

Physiologic Role

Retinoids include vitamin A, its natural and synthetic derivatives, and its metabolites. They exert multiple crucial effects on embryogenesis, cell differentiation, and growth in vertebrates. However, the specific role of the various RARs or RXRs in processes controlled by retinoids in vivo is unclear.
The capacity to differentiate in the presence of RA is lost in two cell lines that carry a RARα protein truncated within the retinoid binding region; one derived from the embryocarcinoma cell line P19, the other from the HL-60 myeloid cell line. Overexpression of the wild-type RARα restores sensitivity to RA in both cell line mutants, suggesting that RARα is physiologically implicated in the regulation of embryonic and myeloid differentiation. However, one must be cautious before accepting this interpretation because: (1) it cannot be excluded that the two RARα mutations interfere, directly or indirectly, with the function of other members of the nuclear receptor family; and (2) there is no unambiguous experimental evidence to support a physiologic role for retinoids in myeloid differentiation.

Biochemical Properties of RARα

The pleiotropic biologic effects of retinoids result from the ability of these molecules to regulate expression of specific genes. Two important properties of RARs are to specifically bind retinoids and DNA.

Ligand-binding properties. RARs bind a variety of different retinoids. All-trans RA (T-RA), a vitamin A metabolite, has very high binding affinity. The binding affinity is similarly high for some retinoids (3,4 didehydro T-RA; 9-cis RA), low for others (13-cis RA). With the exception of T-RA, which binds RXR with very low affinity, the binding affinity of retinoids for RXRs is similar to that of RARs.

DNA binding properties. RARs or RXRs directly bind to specific cis-acting elements (RA-responsive elements [RAREs]) located in the promoter region of RA-target genes. RAREs are polymorphic and contain a direct repetition of two 5'-PuGG(G/T)CA-3' consensus core motifs separated by variable spacing (1 to 5 bp). The spacer, the integrity of the two motifs, and the surrounding bases are critical for the efficiency of RAR binding and transactivation.

Dimerization properties. High-affinity binding of RARs to RARE takes places only if RXR is present. The molecular basis of the RAR/RXR synergism is their dimerization and all combinations of RARs and RXRs heterodimerize. The species that preferentially bind to RAREs under physiologic conditions in vivo are the RAR/RXR heterodimers.

Modular organization. The biochemical properties of RARs have been mapped to specific regions of the RAR molecule. Primary RAR sequences can be divided into six regions (A through F) on the basis of their alignment with the other members of the nuclear receptor superfamily. This subdivision coincides reasonably well with the functional organization of the molecule. The DNA binding domain is made up of two zinc fingers and corresponds to region C. The ligand-binding domain corresponds region E. Regions E and D, respectively, contain the domains for dimerization and nuclear localization. The A/B region has transcriptional activation function (Fig 3).

RARα Regions and Biologic Functions Retained in PML/RARα

As the chromosome 17 break always occurs in RARα intron 2, the RARα component of the PML/RARα protein is the same in all cases of APL and corresponds to regions B to F (Fig 3). Therefore, one would expect PML/RARα to retain the DNA binding, ligand binding, RXR dimerization, and nuclear localization properties of RARα.

DNA Binding

The capacity of PML/RARα and RARα to act as RA-inducible transcription factors has been tested by assessing their ability to regulate the expression of specific RA-responsive target genes (TRE-1K, RAR-α2, RAR-β2, CRABPII, CRBPI) when cotransfected into different target cells (Cos-1, HeLa, HepG2, HL-60). The RA-responsive gene contains a RARE, a minimum promoter region and a reporter gene. Expression of the reporter gene is a measure.
of the capacity of RAR\(\alpha\) and PML/RAR\(\alpha\) to bind the RARE and activate the promoter, assuming that they have equal affinity for retinoids. The results showed that PML/ RAR\(\alpha\)s and RAR\(\alpha\)s have different transactivating properties. The effect on transcription of both PML/RAR\(\alpha\) and RAR\(\alpha\) can be either stimulatory and inhibitory, depending on the promoter and cell type used, either in presence or absence of RA. However, PML/RAR\(\alpha\) is consistently more active than RAR\(\alpha\).\(^{31-34}\) Unfortunately, because cultured promyelocytes and the RAR\(\alpha\) target genes involved in regulating myeloid differentiation are not available, these results do not allow a prediction of the effects of PML/RAR\(\alpha\) on RA-target genes in the APL blasts.

The \(t(15;17)\) causes an interruption in the A/B region of RAR\(\alpha\), so that the A region is lost in PML/RAR\(\alpha\). Because the A/B region contains a transcription-activation function with promoter-context- and cell-type-dependent activity,\(^{65}\) the loss of the RAR\(\alpha\) A region may alter the transcription regulation function of the fusion protein. However, an RAR\(\alpha\) mutant that lacks the A region does not exert the same activity on transcription as PML/RAR\(\alpha\).\(^{32,34}\) Alternatively, the diverse transcription regulation of RAR\(\alpha\) and PML/RAR\(\alpha\) could be caused by the presence of a region with transcriptional regulation function in PML, which may influence the activating or silencing functions of RAR\(\alpha\).\(^{66}\) However, variable portions of PML fused to the GAL4 DNA binding domain did not show any transactivating function of PML on GAL4 reporter gene (our unpublished results, January 1993).

**Ligand Binding**

The RA binding affinity of PML/RAR\(\alpha\) and its specificity for various retinoids are similar to that of RAR\(\alpha\) as shown by Scatchard binding experiments on Cos cells transfected with PML/RAR\(\alpha\) or RAR\(\alpha\) expression vectors.\(^{67}\)

**RXR Dimerization**

PML/RAR\(\alpha\) dimerizes with RXR in vitro and this association results in the binding of PML/RAR\(\alpha\)-RXR heterodimers to RAREs. The PML/RAR\(\alpha\)-RXR association can be visualized in intact cells and, in fact, overexpressed PML/RAR\(\alpha\) and RXR colocalize in Cos cells.\(^{68}\)

**Wild-Type PML Protein**

The information available on PML is limited to its expression pattern, analysis of the primary protein sequence, and its homology with other proteins of known function. The hypothesized functions of PML are based on these homologies.

PML expression is characterized by its ubiquity and its extreme complexity: 13 isoforms have been isolated.\(^{69,70}\) The PML locus comprises nine coding exons. The primary PML transcript can be alternatively assembled into 13 separate transcripts that encode an equal number of PML isoforms. The processing of the PML primary transcript includes the variable assemblage of whole exons, portions of exons, or retained introns. Variable amounts of the PML transcripts have been identified in all histologically diverse human cell lines examined.\(^{69}\)

**Modular Organization of the PML Protein**

On the basis of its homology with other proteins, PML can be regarded as consisting of regions with distinct putative functional relevance (Fig 4).

**The RING finger, the B1 and B2 boxes: The PML putative DNA binding domain.** The N-terminal region of PML contains three clusters rich in cysteine and histidine residues that are retained in all PML and PML/RAR\(\alpha\) isoforms (see below). The first cluster corresponds to a new zinc finger motif (RING motif) that defines a recently recognized family of proteins with functions, where known, that require DNA binding.\(^{70-75}\) These functions include: (1) regulation of development (eg, the XNF-7 gene in Xenopus); (2) regulation of gene expression (eg, RPT-1) that affects the expression of the interleukin-2 (IL-2) receptor gene (our unpublished results, January 1993).

A group of RING genes contains a cysteine/histidine-rich region, termed the B box, which corresponds to an additional putative zinc finger domain.\(^{71,72}\) PML belongs to the RING + B box family, which also includes XNF7, T18, Rfp, Rptl, and the human RNA-binding autoantigen SS-A/ Ro. PML contains two B boxes, which correspond to the second and third cysteine/histidine-rich cluster (Fig 4).

Indirect support for the putative DNA-binding function of PML derives from its nuclear localization. The protein gives a typically speckled pattern within the nucleus by immunostaining.\(^{34,74}\) The region responsible for nuclear localization has been preliminarily mapped within the \(\alpha\)-helix (Fig 4), whereas the region responsible for the speckled distribution has been mapped within the RING finger.\(^{34,68}\)

**The \(\alpha\)-helix and the dimerization domain.** Immediately C-terminal to the fingerlike region is a region that can form multiple \(\alpha\)-helices. A portion of the \(\alpha\)-helix has the potential to assume a coiled-coil configuration and contains four clusters of heptad repeats with hydrophobic amino acids at first, fourth, and eighth positions.\(^{68,75}\) Similar repeats are found in the ligand-binding domain of TR, RAR, and VDR and are considered to be dimerization interfaces.\(^{75}\) The \(\alpha\)-helix without the heptad repeats is variably retained in the different PML isoforms, whereas the fingerlike and coiled-coil regions are retained in all, suggesting that they all have the potential to bind DNA and form homodimers and/or heterodimers.\(^{69}\)

The serine/proline region, the CKII phosphorylation site, and the variable C-termini. Carboxyterminal to the \(\alpha\)-helix is a region rich in serine and proline. It contains several X-S-P-X type repeats that have been identified as the minimum recognition sequence of a serine/threonine kinase and
Putative DNA-binding domain

Dimerization domain

NLS

CKII

Phosphorylation sites

Regions common to all PML isoforms

Variable C-termini

Fig 4. Modular organization of the PML proteins. The diagram shows the modular organization of the various PML isoforms so far identified. All PML isoforms have three regions in common, as indicated: (1) the putative zinc-finger DNA binding domain (the RING domain [R] and the two B boxes [B1 and B2]); (2) the dimerization domain (the coiled-coil region with the four heptad clusters [1, 2, 3, and 4]); (3) a portion of the serine/proline rich (S/P) domain that contains the phosphorylation site for CKII. The non-coiled-coil portion of the α-helix is of variable length in the different PML isoforms. Four alternative C-termini of 41 (PML1 isoform), 259 (PML2), 63 (PML3), and 312 (PML4) amino acids have been identified. The arrows indicate the position of the chromosome 15 translocation breakpoints. The frequency of each bcr is indicated between brackets. NLS, nuclear localization signal.

a casein kinase II (CKII) phosphorylation site⁶⁹ (and our unpublished results, June 1993) (Fig 4). Phosphorylation by CKII is a posttranslational modification shared by many transcription factors and usually associated with modifications in their biologic activity.³⁴ Four alternative C-termini have been identified. Only the CKII site is retained in all PML isoforms.⁶⁹

PML Regions and Functions Retained in PML/RARα

The chromosome 15 breakpoint of the t(15;17) is variably located in three regions of the PML locus. In 90% to 95% of cases, it is equally distributed between intron 6 (breakpoint cluster region 1; bcr1) and intron 3 (bcr3). In the remaining 5% to 10% cases, it is located within exon 6 (bcr2)⁶⁹ (Fig 4). Regardless of the extreme variability of the PML break sites, PML/RARα genes that have the potential to encode for a fusion PML/RARα protein are consistently selected by the leukemia. In bcr1 or bcr3 cases, the 5′ portion of PML intron 6 or 3, respectively, fuse with the 3′ portion of RARα intron 2. During assemblage of the PML/RARα junction in the fusion transcript the chimeric intron is spliced out and the longest PML and RARα open reading frames ORFs become aligned. The operative mechanism of bcr2 is more complex: a cryptic donor site of the retained portion of PML exon 6 and the RARα intron 2 physiologic acceptor site take part in the assemblage.⁶⁹

The Putative DNA Binding Domain

Because of the chromosome 15 breaksite heterogeneity, the retained PML portion differs in each PML/RARα (Fig 4). If the various PML/RARα proteins are compared, it will be seen that the only portions of PML consistently retained in the fusion protein are the fingerlike and the coiled-coil region.⁷⁰

Nuclear Localization of the PML/RARα Protein

The localization of PML/RARα differs from that of PML and RARα. PML/RARα has micropunctated nuclear pattern and PML has a speckled nuclear pattern, whereas RARα is finely dispersed in the nucleus.³⁴,⁶⁸,⁷⁴ Anti-PML antibodies show that APL cells, which express all three proteins, display the PML/RARα-like micropunctuated nuclear pattern, indicating that the fusion protein localization dominates over the two wild-type proteins.⁶⁸ Strikingly, the treatment of APL blasts with RA converts the micropunctuated nuclear pattern to the speckled, PML-like pattern.⁷⁴

Homodimerization and Heterodimerization Potential of the PML/RARα Protein

Analysis of the RA-binding proteins in PML/RARα-expressing cells has shown that the fusion protein is part of multiple nuclear complexes with molecular weights of 600 and 1,200 kD (the apparent molecular weight of PML/RARα is 110 kD).⁶⁷ Although little is known of the proteins that in vivo belong to these complexes, it has been shown in vitro that PML/RARα dimerizes with itself, with PML and, as already discussed, with RXR.⁶⁸

Mechanisms of PML/RARα Action: PML/RARα Is a Multifunctional Protein

PML/RARα can heterodimerize with PML and the nuclear localization of the heterodimer in APL cells is
different from that of PML homodimers. Because it is unlikely that the PML/RARα-PML heterodimers perform the functions of wild type PML, it is conceivable that PML/RARα interferes with PML functions.

PML/RARα also forms homodimers that bind to RAREs with a different specificity than RAR/RXR heterodimers. In addition, preliminary results indicate that PML/RARα homodimers interact with the responsive elements of other nuclear receptors, such as the TR or the VDR (J. Jansen, A. Mahloudi, A. Dejean, personal communication, July 1993) (Fig 5).

Finally, PML/RARα forms heterodimers with RXR. The PML/RARα-RXR complexes are similar to the RAR-RXR dimers in terms of binding activity to RAREs. This is probably caused by the fact that, in the presence of RXR, PML/RARα binds RAREs through one RARα and one RXR DNA-binding domain, as in the RAR/RXR heterodimers, and not through two RARα DNA-binding domains, as in the PML/RARα homodimers. However, the formation of PML/RARα-RXR heterodimers can indirectly influence the functional activity of other nuclear receptors like RARs, TR, and DR, which physiologically dimerize with RXR allowing efficient formation of stable RE-bound complexes (Fig 5).

These multiple biochemical interactions may represent the molecular basis of the biologic activities of the PML/RARα protein (Fig 5). In the presence of low near-physiologic concentration of RA in vitro, PML/RARα acts as a transcription repressor of certain RA-target genes. Furthermore, PML/RARα may also function as a VD signaling antagonist by acting directly on VD-target genes or, indirectly, by sequestering a cofactor, like RXR, essential for D3 receptor activity. As both RA and VD are implicated in myeloid differentiation, these effects may be responsible for the inhibition of differentiation by PML/RARα.

**OTHER TRANSLOCATION PRODUCTS: ABERRANT PML AND RARα/PML PROTEINS**

Because PML/RARα is not the only abnormal product of the t(15;17), one should be cautious in assigning it a solitary role in promyelocytic leukemogenesis (Fig 3).

The chimeric PML/RARα gene encodes not only the PML/RARα protein, but also an aberrant PML protein. The aberrant PML protein is encoded by alternatively spliced PML/RARα transcripts that are assembled in such a way that the longest (ORFs) of PML and RARα are not aligned. Consequently, a stop codon is found a few base pairs 3’ to the PML/RARα junction and the transcript has the potential to encode a C-terminal truncated PML protein. This protein retains the PML putative DNA binding domain and, therefore, the potential to interact with PML target sequences (Fig 3). An aberrant PML protein is present in 100% of APL cases with the t(15;17).

The RARα/PML fusion gene codes an RARα/PML fusion protein. As the RARα/PML is the reciprocal of PML/RARα it contains neither PML nor RARα DNA-binding domains. Instead it includes the RARα A domain, and a variable portion of the PML α-helix tract, phosphorylation sites, and variable C-termini (Fig 3). On the basis of these structural characteristics, it is far from easy to predict whether RARα/PML has a biologic function. Staining of cells that overexpress the RARα/PML fusion protein with anti-RARα A domain polyclonal antibodies has shown that RARα/PML is a nuclear protein. The RARα/PML protein is expressed in 70% to 80% of APL cases.

The t(15;17) is the only known translocation with the potential to encode three abnormal proteins. The fact that, despite the considerable molecular heterogeneity of the t(15;17), the leukemic phenotype selects chromosome
breaks that ultimately lead to the generation of three abnormal proteins, is, to say the least, intriguing. Two other points are worthy of consideration: (1) the t(15;17), unlike other leukemia-associated chromosomal lesions, has never been documented in preleukemic syndromes (15); and (2) the alterations associated with the multistep transformation processes, that are common to other types of myeloblastic leukemia (eg, additional chromosome abnormalities, ras and p53 mutations), have never been seen in APL.6 Could it be that the t(15;17), with its three abnormal proteins, is all that is required to fully transform the APL target cells?

**DIAGNOSIS OF APL**

It is essential that APL be diagnosed accurately and rapidly because it can benefit from specific treatment such as RA and because, if not recognized and treated promptly, it carries the risk of a potentially fatal coagulopathy.81-83

Diagnosis of APL is on the whole easy, certainly easier than diagnosing other myeloblastic leukemias. APL has two distinguishing features: hypergranular morphology and the t(15;17). The granulations, which correspond to primary granules that contain lysosomal enzymes, are pathognomonic for APL and are clearly visible under the light microscopy.84 (Fig 6). However, both markers have limitations. Morphologically, there is a subset of APL, called M3 APL variant (M3v), in which the granules can only be visualized by electron-microscopy.85 Cytogenetically, only 70% to 80% cases display the t(15;17) and good quality bone marrow metaphases cannot always be obtained.24,86 When these two limitations co-exist, diagnosis becomes problematic and Southern blot analysis of the RARα and PML genes is indispensable.

A diagnosis of APL by Southern blot analysis rests on the capacity of the technique to recognize the rearrangements in the PML and RARα genes that occur during translocation.24,28,63-66,87,88 Southern blotting of the RARα gene is of particular value because: (1) It is easy to perform, because it can be limited to the intron 2 region where the RARα consistently breaks.63,65 Only two to three RARα genomic DNA probes are required to identify rearrangements in 100% of cases.63,64,87 (2) Rearrangements are found in both classical cases with a normal karyotype and M3v.24,87 (3) Rearrangements in RARα are also found in the rare APL cases with the 11;17 variant translocation.26,27

APL diagnosis can also be performed by polymerase chain reaction (PCR) analysis of the PML/RARα transcript. However, with respect to Southern blotting, RNA manipulation and PCR technology is probably less appropriate as a routine approach in nonexperienced diagnostic laboratories.

RARα rearrangements or PCR identification of PML/RARα transcripts have the further advantage of being a pathogenetic marker of APL. For example, a number of cases, diagnosed as APL according to French-American-British (FAB) morphologic criteria, have displayed no RARα rearrangements and have failed to respond to RA treatment.89 Therefore, beyond the morphologic concepts, APL should probably be defined as a leukemia characterized by abnormalities of the RARα gene and sensitivity to RA treatment. Leukemias that exhibit no abnormalities in RARα are not APL and will, presumably, be refractory to RA therapy.

**Monitoring the Leukemic Clone During Treatment**

Monitoring of residual disease is particularly important in APL because the incidence of complete remissions (CR) is higher and long-term survival and cure after standard chemotherapy are more frequent in APL than in other myeloblastic leukemias.90-93 When one considers that APL patients achieve a CR easily, and a number will be definitively cured, the value of a test that predicts which CR patients will relapse is obvious.

The presence of a known genetic marker of disease, like the PML/RARα fusion transcript, permits the leukemic clone to be unambiguously distinguished from normal cells. The PCR amplification of the PML/RARα transcript identifies one neoplastic cell against a background of 10⁷ normal cells.94,95 Several groups have set up tests for PCR amplification of the PML/RARα fusion transcript that are easy to perform, need few sets of primers, recognize the transcript in 100% of cases, and show the heterogeneity of the chromosome 15 break during the translocation.89,94-100

Minimal residual disease has been evaluated in a recent retrospective study on 35 APL patients in apparent CR after different therapeutic regimens.100 The APL clone was detected in all cases when the PCR test was performed 1 to 3 months after treatment. Because this “early PCR positivity” did not correlate with response to treatment, it could reflect the presence of differentiated elements likely to disappear in later checks rather than resistant residual blasts.100,101 However, when PCR was performed later after treatment, the prognostic significance of residual disease became clear. Patients who were PCR-positive in two consecutive determinations relapsed within 2 to 4 months, whereas the PCR-negative cases remained free of disease for 3 to 60 months.100 The predictive value of PCR positivity as an indicator of relapse in APL received further support from two independent studies.102,103 Furthermore, residual disease was not detected in nine APL patients in long-term remission (4 to 12 years).96 Although these findings require confirmation in prospective longitudinal studies, they indicate that PCR methodology is a reliable predictor of cure and relapse in APL and that PCR negativity should be considered the therapeutic goal in APL patients. Therefore, PCR can also be applied to assessing the potential capacity of different antileukemic strategies to eradicate the disease. In retrospective studies, RA never induced PCR negativity, bone marrow transplantation (BMT), and the combination T-RA plus chemotherapy did, and chemotherapy alone did so in a percentage of cases.100,102,103

While PCR monitoring studies appear to be clinically relevant in APLs, their value in other leukemias remains controversial. For example, in t(9;22) chronic myeloid leukemia and in t(8;21) AML, patients cells carrying the abnormal bcr/abl or AML1/ETO fusion genes are frequently found after several years of long-term remission.104,105 The persistence of residual leukemia cells in these cases has been interpreted as the consequence of the fact that bcr/abl in CMLs and AML1/ETO in AMLs are not sufficient for the expression of a clinically aggressive disease.
The finding that in APL PCR positivity for PML/RARα correlates with impending relapse and, conversely, long-term remission patients show no detectable transcript, may indicate that PML/RARα is by itself able to confer a fully transformed phenotype to the APL cell.

The M3 Variant APL (M3v)

M3v blasts are microgranular promyelocytes with a distinct folding and lobulation of the nucleus (Fig 6). Ultrastructural investigations have shown the presence of dense granules with an average size significantly less than the 250 nm resolution of light microscopy. Despite these morphologic differences, patients with M3v bear the t(15;17), express the PML/RARα protein, and respond to RA treatment (and our unpublished results, January 1993). However, M3v exhibits other distinctive clinical, phenotypic, and molecular features, suggesting that it evolves genetically from APL.

The hematologic characteristic of M3v at onset is hyperleukocytosis, whereas "classic" APL usually presents with a low leukocyte count. A very severe coagulopathy and death from central nervous system and lung hemorrhage are more frequent in M3v than in APL during the first 10 days after diagnosis.

The immunophenotype of APL is considered to be highly specific (HLA-DR-, CD34-, CD11b+, CD15-, CD9+, CD33+, CD13+). Although the M3v blasts have the same phenotype, they also express the T-cell–associated antigen CD2. In a recent survey of 37 APL cases, 14 of 15 M3v displayed the CD2 antigen, whereas 0 of 22 APL did not (our unpublished results, June 1993).

Molecular analysis of the t(15;17) translocation showed that the incidence of bcr3 type breakpoints is higher in M3v than in APLs.

TREATMENT OF APL

Results obtained with standard chemotherapy in APL differ considerably from those obtained in other myeloblastic leukemias. The incidence of CR is much higher. Sensitivity to anthracyclines (daunorubicin, idarubicin, rubidazon) is also much higher, to the point that it is not clear whether the anthracyclines + AraC combination is superior to anthracyclines alone in APL as it is in other forms of AML. Failure to achieve CR is not normally caused by drug resistance, but rather by death from coagulation disorders during the first days of treatment or from sepsis during postchemotherapy aplasia. Survival is often long-term.

Over the last few years, T-RA has been proposed as an additional tool for treating APL.

T-RA Treatment Produces a High Rate of CR

Since it was first introduced in Shanghai in 1986, about 1,500 patients have been treated with T-RA. A recent review of 565 patients treated at first diagnosis or in relapse gives body to the impression, held since the earliest studies, that T-RA is very effective in inducing complete remission: 84% overall and 95% in patients in whom t(15;17) was documented by cytogenetic or molecular analysis. The hematopoietic reconstitution in CR patients is polyclonal. The response rate is also high in patients resistant to chemotherapy, whereas results are less favorable in second or subsequent relapse. Failure to reach CR with T-RA is generally due to early death rather than to leukemia resistance.

CR Is Reached Without a Phase of Bone Marrow Aplasia

T-RA–treated patients achieve CR in 1 to 3 months (median 35 to 45 days) without suffering severe bone marrow depression. Patients generally continue treatment during this period and in most cases the dose is 45 mg/m²/d.

T-RA Is Also Effective for Treating APL-Associated Coagulopathy

APL is associated with severe coagulation disorders, characterized by bleeding diathesis, which is present in 80% of patients at onset, usually becomes more serious during chemotherapy, and may also appear in the other 20% of patients. Bleeding diathesis in APL has been attributed to disseminated intravascular coagulation (DIC) and excessive fibrinolysis, which are the result of the release of procoagulants and tissue plasminogen activator from neoplastic promyelocytes during senescence and chemotherapy-induced cell lysis. Traditional treatment for APL-associated coagulopathy during chemotherapy is fresh-frozen plasma transfusion, intensive platelet support, and tranexamic acid, whereas the value of heparin is debatable. T-RA rapidly resolves the coagulopathy within the first 48 hours of treatment, before any morphologic effect is seen. T-RA probably acts by reducing the mass of neoplastic promyelocytes without provoking cell lysis.

T-RA Treatment Is Generally Well Tolerated

Toxicity caused by treatment is, in most cases, modest. Dry skin and mucosae are the most common; cheilosis, nasal stuffiness, and itching less frequent; whereas other symptoms such as headache, flaxing, clogged ears, cervical and tonsilar lymphadenopathy, bone pain, and arthralgias are rarely seen. The side effects are mostly short-term and easily controlled by appropriate symptomatic therapy. Biochemically, treatment is sometimes associated with hypertriglyceridemia, and an increase in serum aminotransferase, alkaline phosphatase, and bilirubin. Intracranial hypertension, with cerebral pseudotumor, occasionally occurs, primarily in pediatric patients, and can be dose-limiting.

The Only Severe T-RA Side Effect is the So-called "Retinoic Acid Syndrome," But This Can Be Treated or Prevented

This syndrome, which occurs in about a quarter of patients, usually early in the course of treatment (between the second day and the third week), is clinically very similar to the capillary leak syndrome observed in patients treated with various cytokines (fever, respiratory disease, radiographic pulmonary infiltrates, pleural effusions, renal impairment and, in some patients, cardiac failure). It is more frequent in APL patients who initially present with high tumor burden or develop rapid increase in the leuko-
cyte count during treatment (myeloblasts, promyelocytes, cells in various stage of differentiation and neutrophils). If not promptly treated, the retinoic acid syndrome can lead to death from progressive hypoxemia and multiorgan failure. There is no agreement as to the best treatment. Some find steroid therapy efficacious, others prefer to prevent its onset by treating all patients that tend toward leukocytosis with cytoreductive therapy (high- or intermediate-dose chemotherapy, leukapheresis). Administration of standard chemotherapy (AraC + daunorubicin) when the white blood cell count of patients in treatment with T-RA rapidly increases is effective in preventing the retinoic acid syndrome. High doses of corticosteroids early in the course of the syndrome have led to prompt improvement in some patients.122

The etiology of the retinoic acid syndrome is unknown. It may be a consequence of PML/RARα activity on ICAM-1, an adhesion molecule that regulates homotypic and endothelial cell adhesion of leukocytes.125 We have data (unpublished, August 1993, in collaboration with A. Pinto, Centro Riferimento Oncologico, Aviano, Italy) to show that PML/RARα induces a marked, RA-dependent, increase in ICAM-1 expression in maturing myeloid cells both in vivo and in vitro.

**Duration of the Hematologic Remission After T-RA Is Generally Brief**

Few patients are still in CR 1 year post T-RA therapy; mean duration reported in different studies varies from 1 month to 2 years.1,3,112-118 Randomized studies that compare RA plus chemotherapy with chemotherapy alone are in progress and should show whether RA plus chemotherapy is superior to chemotherapy alone in terms of remission frequency, toxicity, and treatment of complications and survival rate.124,126 A pilot study on 26 newly diagnosed APL patients shows that T-RA followed by intensive chemotherapy (daunorubicin plus AraC) slightly increases the rate of CR and reduces the relapse rate.124

**Post T-RA Therapy Relapse Is Resistant to T-RA**

Despite the fact that de novo resistance to T-RA is very rare, relapsing patients are invariably resistant to further treatment with T-RA. However, the acquisition of T-RA resistance does not imply the acquisition of chemotherapy resistance.1,3,112-118

**Choice of Treatment in APLs**

Two treatment strategies are successful in inducing CR in APLs: T-RA (>90%) and anthracycline-based chemotherapy (75% to 80%). As intrinsic resistance to both T-RA and anthracyclines is very low in APLs, the effect T-RA exerts on the coagulopathy is probably responsible for these differences. Although, in terms of cure, anthracyclines are superior (35% to 45%) to T-RA (practically 0%), the combination of the two induces PCR negativity of PML/RARα and appears to increase the CR and cure rates of those obtained with either treatment alone.124,128 Moreover, the use of high-dose chemotherapy after T-RA is in keeping with principles of modern chemotherapy, ie, to administer maximum doses when the tumor mass is the lowest possible, and to use agents that do not induce cross-resistance.

A major unanswered question is: what strategy should be followed in APL patients who have already entered CR? There is no agreement on the optimal postinduction chemotherapy. Whereas intensive consolidation chemotherapy seems to be useful,23 the value and modality of maintenance chemotherapy is still in doubt, but studies are underway that should provide an answer (Gimema Italian cooperative group; in progress). To date no studies have compared postinduction chemotherapy with allogenic BMT in APLs. However, because 35% to 45% of APL CR patients are in fact already cured, the choice of submitting all patients to the risk of BMT is, at best, debatable.

PCR analysis of APL residual disease should be able to solve this problem. If its value in predicting cure will be confirmed, PCR should be considered fundamental for identifying those CR patients who have not obtained cure and to rapidly evaluate the eradication potential of the various intensification regimens (BMT or high-dose chemotherapy) and select the most appropriate one.

**Mechanism of RA Sensitivity**

The cellular mechanism of T-RA sensitivity in vivo seems to be the terminal differentiation of the APL blasts. This interpretation is in agreement with the fact that APL blasts differentiate in the presence of T-RA in vitro and that the in vivo response can be predicted by in vitro differentiation tests.114,117 It is also supported by the findings of: (1) absence of bone marrow aplasia during treatment; (2) appearance, during treatment, of cells having the morphologic characteristics of maturation stages intermediate between promyelocytes and neutrophils; (3) presence, during treatment, of PML and RARα rearrangements in peripheral blood neutrophils that disappear after treatment.128

Although the molecular mechanism awaits formal proof, there is experimental evidence that PML/RARα underlies the sensitivity of the promyelocytic blasts to T-RA. Indeed, there is a strict correlation between the expression of the PML/RARα fusion transcript and response to treatment.89 In addition, PML/RARα increases the sensitivity to RA in vitro,92 and clones of the APL NB4 cell line become T-RA-resistant when they lose PML/RARα expression.129

Any attempt to explain the biochemical mechanism through which PML/RARα mediates differentiation in the presence of RA runs into the same perplexities encountered in trying to describe the role of PML/RARα in the induction of the differentiation block. As previously mentioned, the PML/RARα fusion proteins retain both the RARα DNA and retinoid binding domains and, therefore, they could directly influence the RARα-dependent endogenous pathway that controls terminal myeloid differentiation. Transactivation experiments have shown that, in certain experimental conditions, PML/RARα overstimulates the expression of RARα target genes when RA is present.31-34 This PML/RARα function might explain RA sensitivity of APL blasts.

Alternative explanations for sensitivity to RA are based on the claim that PML/RARα is localized, at least in part, in the cytoplasm.34,70 The cytoplasmic PML/RARα would constitute a barrier for RA, that can be bypassed by high
concentrations of RA. However, it cannot be excluded that RA may exert its effect on PML/RARα by triggering the release of the hypothetical dominant negative action over PML. It should be kept in mind that the interaction between RA and the RA binding domain of PML/RARα could cis-activate the PML portion of the fusion protein, and that RA changes the PML/RARα nuclear localization pattern to one that is indistinguishable from that of PML.76

Sensitivity to RA could, instead, be an intrinsic property of promyelocytes and, therefore, also of leukemic promyelocytes. The promyelocytes that occupy the bone marrow in congenital agranulocytosis are, in fact, sensitive to RA.130 The translocation products would be involved in RA sensitivity only to the extent that they cause a differentiation block at the promyelocytic stage.

**Mechanism of RA Resistance**

Resistance to RA that develops post-therapy at the time of relapse can be partially explained on a pharmacokinetic basis. As treatment proceeds, the bioavailability of the drug is lowered by an as yet unidentified mechanism.38,131 However, in most cases of post RA therapy relapse, the promyelocytic blasts are resistant in vitro to the differentiation action of RA, which suggests that during treatment they have accumulated mutations in molecules involved in the response to RA (C. Chomienne, personal communication and our unpublished results, May 1993). Candidate molecules are those dedicated to the intracellular transport of RA (cellular-retinoic-acid-binding proteins (CRABP)) and those involved in the response to RA (PML/RARα, RARα, RXR). RA-resistant subclones of the NB4 cell line were shown to have lost the expression of the PML/RARα protein, suggesting that an alteration of the fusion protein is associated to retinoic acid resistance.129

**FUTURE PROSPECTS**

It is apparent from this review that there is still much to be learned on the molecular mechanisms through which PML/RARα induces a differentiation block and through which APL cells are sensitive to RA. In vivo, PML/RARα could interfere with many of the signaling pathways activated by hormone receptors such as RARs, RXR, VDR, and TR. But how they regulate terminal differentiation and interfere with other pathways known to be implicated in hematopoietic regulation (eg, the cytokine signaling pathways) cannot, as yet, be explained. Clarification of these issues should provide a picture of the interdependence among multiple mechanisms that regulate differentiation. In the end, they should also be able to establish if other, and which signaling pathways, can be activated to release or bypass the APL differentiation block or synergize with RA. Within the same framework, much should be learned about the PML/RARα-activated pathways that regulate cell survival, their relation with those that regulate differentiation, and their dependence on RA or other effectors.

PML/RARα influences differentiation at more than one level. The model derived by the various biologic activities of PML/RARα would predict two leukemic cell populations: one less differentiated than promyelocytes, with self-renewal potential and probably the true leukemic reservoir; and another of committed precursors with promyelocytic morphology (Fig 2). These two populations may not be equally sensitive to RA, which would explain some of the characteristics of the response to RA therapy (eg, failure to eradicate the disease).

Studies already underway should lead to new therapeutic strategies for treating APL. They are based on the fact that the PML/RARα junction creates a new tumor-specific sequence in APL. It has been shown that the PML/RARα fusion protein contains an antigenic site, not present in the normal parent molecules and recognizable by human CD4+ T-lymphocyte clones. One of these clones had cytotoxic activity against autologous cells expressing the PML/RARα protein.132 These findings provide the background for future adoptive immunotherapy investigations. Furthermore, the PML/RARα transcript is a putative target for therapy based on the use of antisense oligonucleotides. Similar therapies are being tested for other disorders (eg, chronic myeloid leukemia) in experimental animals.

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