of the candidate genes in zebrafish (Danio rerio). The strategy here was to attempt to predict functions of the human proteins using a “reverse genetic” screen in which expression of the fish orthologs was “knocked down” and the phenotype, or lack of it, was determined in an induced thrombus model. Zebrafish have been touted as unique surrogate systems for reverse genetic analysis, and their potential use in specific studies of hemostasis is supported by similarities between blood cells and humoral coagulation pathways in humans and this fish species.

The fishing expedition looking for new functional proteins was a success, and O’Connor et al netted 4 factors with roles in laser-induced thrombosis (2 promoters, 2 modulators) in the surrogate zebrafish system. The phenotypes presumably indicate activities of the fish orthologs when they are expressed on circulating thrombocytes, but a caveat is that knockdown by morpholino antisense technology, which was the approach used, is not cell specific. Thus, one or more of the proteins could also have activities on endothelial cells or other cell types. Nevertheless, the results of the reverse genetic screen are enticing enough to merit evaluation of the candidate proteins in human platelets—where their functions remain in question—and, again with appropriate caveats, in mouse knockout models.

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**CLINICAL TRIALS**

Comment on French et al, page 4512

**Old drug, new lessons**

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In this issue of *Blood*, French and colleagues use several genome-wide approaches and report that acquired genetic variation has a stronger impact on methotrexate polyglutamate accumulation in acute lymphoblastic leukemia cells than inherited genetic variation.

This paper by French et al is among the first to combine high-throughput analyses of malignant cells for acquired genetic variation (mRNA expression and DNA copy number variation) and of normal cells to detect inherited genetic variation (DNA single nucleotide polymorphisms and DNA copy number variation) in the same patients. A relatively large cohort of children with acute lymphoblastic leukemia (ALL) was tested, and methotrexate polyglutamate (MTXPG) accumulation was determined ex vivo in samples obtained 42 to 44 hours after the patients had been treated with single-agent MTX at 1 g/m² given intravenously over 4 or 24 hours. Acquired genetic variation was assessed in leukemic cells obtained at diagnosis while inherited genetic variation was studied in DNA extracted from whole blood sampled after the patients achieved complete remission.

This study is important for several reasons. First, it demonstrates the importance of characterizing malignant cells themselves in order to predict or explain their sensitivity to a particular drug. Apparently, the biology of malignant cells is more important in this respect than inherited factors, which, for instance, influence pharmacokinetics. Second, it identifies novel genes, especially on chromosomes 10 and 18, which seem important in explaining variation in MTXPG accumulation. Third, the paper contains a wealth of information on the relevance of individual chromosomes and genes regarding MTX accumulation. Finally, it shows that combining information on acquired and inherited genetic variation can be useful, since this analysis identified 7 genes that had the strongest impact on MTXPG accumulation.

Inevitably, the study also has some weaknesses, and several questions remain to be answered. While 248 patients were eligible for the study, actual characterization was limited to 145 patients for mRNA expression, 82 patients for leukemia cell DNA copy number variation, and 144 patients for inherited DNA genotyping. The authors demonstrate that this did not result in a statistically significant selection bias, but some impact of the subset of patients available for each assay type cannot be excluded. Moreover, patient numbers limited the power of the study to detect smaller but still relevant correlations between genetic variation and MTXPG levels. An open question is whether the genes that explained variation in MTXPG accumulation have a causal role in determining the clinical response to MTX treatment. After all, this was a correlational study and moreover, MTXPG levels are a surrogate for sensitivity or resistance to MTX, although other studies demonstrated a correlation between MTXPG levels and both in vitro and in vivo efficacy of MTX. It also would be interesting to know what the study results would have been in case of a higher dose of MTX (eg, 5 g/m²), a dose now being used in many protocols. French et al indeed report that MTXPG levels differed between the 4- and 24-hour infusion schedules, and they cite literature that reported that gain-of-chromosome 21 was associated with increased MTXPG accumulation, but only in the case of treatment with MTX at 180 mg/m² given orally over 36 hours and not in the case of 1 g/m² given as a 24-hour infusion. Similarly, the findings explain up to two-thirds of the variation in MTXPG levels, but what about the remaining one-third? Finally, the authors do not provide data on toxicity of MTX. The current analysis might suggest that characterization of inherited genetic variation is of limited value. However, such variation is more
likely to explain side effects of specific treatment elements and is important as well. It was beyond the scope of this study and will be extremely challenging, but one wonders if similar findings will be made if minimal residual leukemic cells are studied.

Meanwhile, this study encourages similar efforts focusing on other drugs. Given the large interindividual variation in drug sensitivity, the complicated biological processes that determine their antileukemic activity and toxicity, and their importance in the treatment of childhood acute leukemias, obvious candidates are cytarabine, l-asparaginase, and glucocorticoids. The understanding of the efficacy of other drugs and in a wide range of cancers will also benefit from this type of integrated research. In the longer term, this approach is not feasible for routine use in multicenter clinical studies. Therefore, customized chips should be developed, which likely need center clinical studies. Therefore, customized chips should be developed, which likely need
to be disease- and treatment-specific. Ultimately, this type of research will enable more individualized and tailored chemotherapy of cancer, aiming at more effective and less toxic therapy.

**IMMUNOBIOLOGY**

Comment on Zapata et al, page 4595

**TRA3 in B cells: too much, too little, too bad**

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In this issue of *Blood*, Zapata and colleagues investigate a novel transgenic mouse model overexpressing TRAF3 in B cells and surprisingly find plasmacytosis and hypergammaglobulinemia, even though TRAF3 is reported to be a tumor suppressor gene in MM.

Homozygous inactivation of tumor necrosis factor (TNF)–receptor–associated factor 3 (TRA3) was recently reported in 13% of multiple myeloma (MM) patients. It has been found to be a negative regulator of NIK, which, as expected, is elevated in MM with TRAF3 inactivation. NIK is a key enzyme regulating processing of NFKB2 p100 to p52 that dimerizes with relB and results in transcriptional activation of NFkB targets. Highlighting the importance of NIK stabilization, additional mutations with similar functional consequences occur in MM, including biallelic deletions encompassing both cIAP1 and cIAP2, as well as biallelic deletions of TRAF2, all contributing together with TRAF3 to form a NIK negative regulatory complex. Based on constitutive p100 processing, and the presence of nuclear p52-relB complexes in TRAF2 or TRAF3 deficient B cells, they have been described as “inhibitors” of the alternative NF-kB pathway. Whereas tumors with dysregulated overexpression of TRAF3 have not been reported, amplification and overexpression of cIAP1/2 occurs in various tumors and has been shown to be critical in liver cancer.

Consistently, mice with conditional inactivation of TRAF3 in B cells develop splenomegaly and lymphadenopathy due to B-cell expansion, and hypergammaglobulinemia with increased plasma cell numbers. On the other hand, the TRAF3 transgenic mice described in the current report have lower levels of NIK with splenic hypotrophy and reduced numbers of B cells. The unexpected finding, however, is that elevated TRAF3 in these mice also results in hypergammaglobulinemia and plasmacytosis. Zapata et al have shown that this phenotype is not due to increased B-cell proliferation, but suggest it may be the result of heightened Toll-like receptor (TLR)–mediated B-cell differentiation. Interestingly, the mice develop a high incidence of solid tumors (67%) with age, primarily squamous cell carcinoma of the tongue, which the authors suggest may result from the chronic inflammation present in these mice. As noted, this represents the first model of spontaneous carcinogenesis initiated by B-cell mediated chronic inflammation. A potential limitation of these studies is that only a single transgenic founder line was examined in detail, and the phenotype may potentially relate to the site of transgene insertion in this founder.

Additional studies of these fascinating mice will be required to better understand the mechanisms underlying the many surprising observations these mice afford.

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**TRA3 in B cells: too much, too little, too bad**

TRAF3 is a critical regulator of plasma cell homeostasis. TRAF3 is an adaptor that recruits a TRAF2, cIAP1, and cIAP2 E3 ubiquitin ligase complex to NIK, leading to rapid turnover in resting B cells. Low levels of TRAF3 lead to NIK stabilization, activation of the alternative NF-kB pathway, and plasma cell survival. High levels of TRAF3 in transgenic B cells result in reduced B-cell numbers, but plasmacytosis by an unresolved mechanism, perhaps related to increased plasma cell differentiation.
Old drug, new lessons

Gertjan J. L. Kaspers