from one of these families is shown in lanes 1, 2, and 3 of Figure 2 from the original paper.\cite{lobo2000}
To further confirm that these amplification products were polymorphic alleles, we directly sequenced from the PCR products of the 2 most common alleles (J.C.W., unpublished work, August 1998). We did not sequence the rarer allele, as there were no homozygous samples. We also confirmed that the polymorphic alleles were in Hardy-Weinberg equilibrium, and it would be unlikely that we would see this ratio if we were amplifying random bits of the genome. Finally, repeat remission samples for some of the patients existed and, where possible, these also were amplified (unpublished work). Again, we found no discrepancies. For these reasons we believe that our data are sound.

van Schooten et al do not suggest any alternative explanations for the discrepancies between the 2 sets of data. Racial differences in the normal population groups or sample size may account for some of the inconsistency. An alternative explanation is that van Schooten et al have not resolved the PCR products sufficiently. This would result in the 2 most common alleles appearing to be the same size and only the larger allele distinguishable as a polymorphism. Consequently, the heterozygosity frequency would appear much lower than it actually is, since the larger allele is much rarer. In our hands the frequency of the rarer allele was 0.07, and this is consistent with the frequency of 0.06 observed in van Schooten et al’s control samples. Poor resolution of the PCR products may also explain why no microsatellite instability was observed in the acute lymphoblastic leukemia samples.

The authors also suggest that the loss of heterozygosity (LOH) we saw was purely artifactual. It is worth pointing out that we were able to confirm LOH in 2 of these samples by fluorescence in situ hybridization analysis. Since then, there have been a number of papers describing LOH at the MLL gene locus using various different methods.\cite{haidar1999, takeuchi1999, takeuchi2000, matthew2000, matthew2000}
van Schooten et al’s suggestion that there is no LOH at this polymorphism is not backed up by their data: they see very little heterozygosity, and it is not possible to detect loss of a homozygous allele when using only a nonquantitative PCR-based technique.

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References


To the editor:

The −1C>T mutation in the annexin A5 gene does not affect plasma levels of annexin A5

González-Conejero and coworkers describe how the −1C>T mutation, located in the Kozak sequence of the annexin A5 gene (formerly named annexin V), reduced the risk of myocardial infarction in young patients.\cite{gonzalez2000} They postulated and showed supporting evidence that −1C>T in the Kozak sequence of the annexin A5 gene increased translation, resulting in higher plasma levels of annexin A5 in T allele carriers. These findings do not support the Kozak context rules as reported by Marilyn Kozak.\cite{kozak2000, kozak2000}
She furthermore had some practical comments about the reliability of the in vitro translation assay used in the study.\cite{kozak2000}

Our concerns are related to the determination of the plasma annexin A5 levels in citrated plasma. González-Conejero and coworkers used the enzyme-linked immunosorbent assay (ELISA) technique of Diagnostica Stago.\cite{gonzalez2000} This ELISA allows only the measurement of free nonbound annexin A5. As annexin A5 binds with high affinity to negatively charged phospholipids in the presence of calcium,\cite{gonzalez2000} addition of citrate will not disturb the binding efficiently and thus, the levels of annexin A5 measured in citrated plasma will be underestimated. The annexin A5 levels are significantly higher in EDTA (ethylenediaminetetraacetic acid) plasma.\cite{gonzalez2000}
In all our studies performed so far, we measured only increased annexin A5 levels in EDTA plasma. This might indicate that the annexin A5 levels in citrated plasma are not biologically active or are released out of the residual platelets due to freeze-thawing the plasma.

To determine whether T allele carriers indeed have increased levels of circulating annexin A5, we measured the annexin A5 levels in citrated and EDTA plasma of 40 healthy volunteers and linked the results to the −1C>T mutation. All plasma samples were obtained by centrifugation of citrated and EDTA blood for 10 minutes at 4000g. The plasma samples were stored at −80°C.

Our results show significantly higher annexin A5 levels in EDTA plasma compared with citrated plasma (1.15 ± 0.56 vs 0.95 ± 0.28 ng/mL). Furthermore, among these 40 healthy volunteers,

Figure 1. Annexin A5 levels in C allele carriers and carriers of the −1C>T mutation. Annexin A5 levels were measured with the ZymoTest Annexin A5 ELISA (Hyphen Biomed, Andresy, France), and the polymorphism was determined by Ncol restriction fragment-length polymorphisms (RFLP). The mean annexin A5 levels are expressed (± SEM).
no differences in annexin A5 levels (both in citrated and EDTA plasma) were observed between T allele and C allele carriers (Figure 1). Our measurements are in contrast to the results of González-Conejero et al, who showed increased levels of annexin A5 in plasma of T allele carriers. The prevalence of the mutation in our control group was comparable to the prevalence in the Mediterranean population (26%).

We have shown that the −1C>T mutation does not result in elevated plasma annexin A5 levels. More studies have to be performed to understand the reduced risk of myocardial infarction in young patients carrying the T allele in the Kozak sequence of the annexin A5 gene.

Response:

Annexin V polymorphisms, plasma levels, and myocardial infarction

Studies about common polymorphisms give conflicting results when analyzing both their functional role and their clinical relevance. For every study that finds a positive association with a particular polymorphism, there are several reporting no effect or the opposite. As expected for genetic changes present in more than 1% of the population, common polymorphisms would unlikely have a strong effect. Thus, even minor modifications in selection of samples, design of the study, environmental factors, or genetic background of different populations might modify the functional or clinical consequences associated with a single polymorphism. In a recent study, we found the −1T allele of the annexin V (ANV; highly prevalent in the white Mediterranean population [23%]) associated with increased circulating levels of this protein in citrated samples. This allele related to increased translation of this protein when using in vitro systems containing either whole ANV cDNA or mini-cDNA generated by genomic polymerase chain reaction. In agreement with these results, we found that the −1T allele conferred protection against premature myocardial infarction. van Heerde and coworkers have argued against some points of our findings a positive association with a polymorphism. In the annexin V Kozak sequence (−1C>T) increases translation efficiency and plasma levels of annexin V, and decreases the risk of myocardial infarction in young patients. Blood. 2002;100:2081-2086.

Kozak M, Neufeld E. Not every polymorphism close to the AUG codon can be explained by invoking context effects on initiation of translation. Blood. 2003; 101:1202.


Agranulocytosis unresponsive to growth factors following rituximab in vivo purging

Rituximab, a chimeric monoclonal anti-CD20 antibody, has been successfully used to in vivo purge CD20+ tumor cells during mobilization for high-dose therapy with autologous stem cell transplantation.\(^1\) \(^6\) Posttransplantation neutropenia has been observed in as many as 25% of patients, although, to date, all reported patients have responded to growth-factor administration. In this report, we describe a case of agranulocytosis refractory to high-dose stem cell growth factors and ultimately responsive to cyclosporine following autologous stem cell transplantation for non-Hodgkin lymphoma (NHL) purged in vivo with rituximab.

A 46-year-old man who presented with stage IV lymphocyte-predominant Hodgkin disease was treated with standard chemotherapy and achieved a brief partial response. Review of the pathology resulted in modification of the diagnosis to diffuse large B-cell lymphoma, T cell and histiocytic rich. Standard CHOP chemotherapy resulted in another brief partial response. At the time of disease progression, in preparation for an autologous stem cell transplantation, the patient received and responded to 2 cycles of ifosfamide, mesna, carboplatin, and etoposide (Figure 1). He underwent successful chemomobilization therapy that included 2 doses of 375 mg/m\(^2\) rituximab, 7 days apart. He received 3.3 \(\times\) 10\(^6\) CD34 cells/kg following a myeloablative conditioning regimen. Trilineage engraftment occurred by day 9 after transplantation. Rituximab was administered on days 30 and 37 without complication. On day 77, his total white blood cell (WBC) count was 5000 mm\(^3\) with 86% neutrophils, hemoglobin level 12.0 g/dL, platelet count 132 mm\(^3\). On day 122, the patient had a WBC of 1000/mm\(^3\) with 0.0% neutrophils, hemoglobin level 13.0 g/dL, platelet count 185 000 mm\(^3\). A bone marrow biopsy performed on day 128 showed hypocellular bone marrow with little maturation of the myeloid series and no evidence of disease relapse. Dysplastic features were not observed despite a new cytogenetic abnormality, del(20)(q11.2). An extensive evaluation for an infectious etiology was negative. Autoantibodies against granulocytes were not detected.

Possible offending agents were discontinued. Granulocyte colony-stimulating factor (GCSF) began followed by 2 doses of intravenous immunoglobulin and granulocyte-macrophage colony-stimulating factor (GM-CSF). Despite these maneuvers, absolute neutropenia persisted for longer than 65 days. Within 5 days of initiating therapy with cyclosporine (200 mg orally twice a day), the neutrophil count returned to normal levels. GM-CSF and GCSF were tapered with no decrement in neutrophil count. The patient continues on cyclosporine with stable neutrophil counts 14 months later. Attempts at dose reduction to 25 mg orally twice a day have persistently resulted in relapsed neutropenia suggesting ongoing autoimmune destruction of myeloid precursor cells.

This is the first reported case of agranulocytosis associated with rituximab therapy that was refractory to growth-factor support but responsive to cyclosporine. The efficacy of cyclosporine suggests a T-lymphocyte–specific cellular response against autologous myeloid precursors. Although cyclosporine has been used in the post–autologous stem cell transplantation setting to induce autologous graft-versus-host disease (GVHD), there are no reports of its use in the post–autologous transplantation setting for the treatment of cytopenias. Del(20)(q11.2) has been reported with myelodysplastic syndromes (MDSs) related to high-dose therapy and autologous transplantation for patients with non-Hodgkin lymphoma, and successful immunosuppressive therapy with cyclosporine for MDS has been reported.\(^7\) \(^9\) This patient, however, had no other features suggestive of MDS. This single case experience suggests that cyclosporine be considered in patients with rituximab-associated neutropaenia not responsive to GCSF.

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