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*J Immunol* 2001; 167:2457-2458; doi: 10.4049/jimmunol.167.5.2457

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Technical Note: Linkage Disequilibrium and Disease-Associated CTLA4 Gene Polymorphisms

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CTLA4 and CD28 are important regulators of T lymphocyte activation. Gene region 2q33 carrying genes for both CTLA4 and CD28 has been shown to be linked to many autoimmune diseases. Disease associations with particular CTLA4 gene polymorphisms have been reported. Recently, first lines of evidence emerged for functional effects of CTLA4 gene polymorphisms. Two independent studies reported a reduced inhibitory function of CTLA4 in individuals with certain CTLA4 genotypes: those with a high number of microsatellite repeats in one study and those with allele +49G in exon 1 in the other one. We analyzed the strength of linkage disequilibrium between the three known CTLA4 polymorphisms among 577 independent chromosomes. Our results show that the polymorphisms previously suggested to be the functional risk factors nearly always occur together in a very frequent haplotype. Due to this strong linkage disequilibrium, we conclude that the previous reports studying merely a single polymorphism could not distinguish which variation actually caused the functional difference. Hence, either mutagenesis approaches or studies with data on all linked polymorphisms are still needed to determine the genuine functional risk polymorphism in this gene region. The Journal of Immunology, 2001, 167: 2457–2458.

Genetic linkage or association between several autoimmune diseases and CTLA4 gene region on chromosome 2q33 has recently been reported in a number of studies (1). As CTLA4 is an important negative regulator of T cell activation, this receptor is a plausible candidate for a susceptibility gene in diseases with T cell mediated pathogenesis. Ongoing research in the gene region has not yet revealed consistent evidence for functional risk alleles, but two recent studies by Kouki et al. (2) and Huang et al. (3) presented important data that particular polymorphisms could cause reduction of the CTLA4 inhibitory function.

Three genetic polymorphisms in the CTLA4 gene (Fig. 1) have been reported so far, all of which may be able to alter the function or expression of the molecule. For example, in Graves’ disease, all three polymorphisms have been reported to show association with the disease in case-control studies (1). A single nucleotide polymorphism at position −318 (hereafter −318C/T) is located in the promoter region (4). A significant increase in the C/C homozygosity among Graves’ disease patients was reported in a Danish case-control study (5).

Another polymorphism, A/G variation at position +49 (+49A/G) in the first exon of the gene leads to threonine to alanine change in the leader peptide. Association of allele +49G in Graves’ disease has been found in several studies (6, 7). Recently, Kouki et al. (2) studied the CTLA4 expression and T cell proliferative responses in Graves’ disease patients and healthy controls genotyped for +49A/G. Importantly, they found correlation of +49G/G genotype with reduced inhibitory function of CTLA4, and suggested that this particular polymorphism could be the actual disease-associated allele.

A similar effect on CTLA4 function has been suggested for the third polymorphism, microsatellite AT-repeat in CTLA4 (CTLA4 (AT)n), which is located in the 3’ untranslated region of the last exon and has at least 20 alleles (8). Alleles with long repeat regions, in particular the *99 allele (i.e., allele with the apparent length of 99 bp in our genotyping conditions, equal to 106 bp in many other studies), have been found to be associated with Graves’ disease in two studies (9, 10) and with another autoimmune disease, myasthenia gravis (11). Recently, Huang et al. (3) reported a decrease in the CTLA4 inhibitory function in myasthenia gravis patients carrying these long repeat alleles. In addition to higher proliferation state of T cells, the patients with the long alleles had elevated levels of serum IL-2 sRα and higher telomerase activity, both markers for T cell activation, than patients carrying the shortest alleles.

As the two studies describing the functional effects of the polymorphisms analyzed only one of the polymorphisms in relation to the CTLA4 function, we wanted to study the occurrence of the genetic polymorphisms in the CTLA4 gene in more details. In particular, we investigated the gametic association, or linkage disequilibrium, of the three genetically polymorphic sites in the CTLA4 gene to see whether the simple association analyses are able to reveal which polymorphism in fact causes the observed effect on the CTLA4 expression. A total of 577 independent haplotypes of the three polymorphic sites were constructed from analysis of 156 nonrelated Finnish families with celiac disease, using the Genehunter 2.0 program (Whitehead Institute for Biomedical Research, Cambridge, MA) (12). Exact test of linkage disequilibrium between pairs of polymorphic sites and standardized disequilibrium value D’ for each allele pair were calculated using the Arlequin program (13). Values of D’ range from −1.0 to complete disequilibrium, 1.0, through 0 stating for no linkage disequilibrium between the pairs. We recently reported significant genetic linkage between the CTLA4 gene region and celiac disease (14), but none of the three markers showed allelic association with this disease (Ref. 14 and our unpublished results). In addition, sequencing of the promoter and all exons of the CTLA4 gene from five Finnish

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2 Abbreviation used in this paper: CTLA4(AT)n, microsatellite AT-repeat in CTLA4.
individuals did not reveal any additional polymorphic sites (our unpublished results), indicating that no other common polymorphisms may be present in the gene.

Extremely strong linkage disequilibrium was found between all pairs of three polymorphic sites (exact \( p = 0.00 \)). Forty-one different allelic combinations, or CTLA4 gene alleles, were observed among 577 haplotypes as shown in Table I. Two haplotypes, −318*C; +49*G; CTLA4(AT)\textsubscript{a}*81 and −318*C; +49*G; CTLA4(AT)\textsubscript{a}*99, were found to be the most common ones in Finland with frequencies of 30 and 37%, respectively. What is important for the present report, 97% (310/321) of +49*G alleles occurred with long microsatellite alleles (*93*–*125*); mainly with the allele *99 reported to associated with Graves’ disease (D\(^2 = 0.95 \)). In other words, the polymorphic site reported by Kouki et al. (2) as well as the long microsatellite alleles reported by Huang et al. (3), to affect the function of CTLA4, occurred together almost with no exception. Unfortunately, these authors investigated only one of the two polymorphisms, instead of investigating more detailed haplotype combinations. According to the present results these two polymorphisms very often co-occur and it seems impossible to dissect by a single marker association analysis which polymorphism, if either of them, actually explains the observed decrease in the inhibitory function of CTLA4 in T cell activation. The question could only be addressed either by mutagenesis approaches or by haplotype, or at least genotypic, data on all linked markers, which allows the phenotypic comparison of individuals positive for the suggested risk allele at 1 polymorphism and either positive or negative for the risk alleles at the others.

It is of note that the level of linkage disequilibrium in regions carrying genes for rare monogenic diseases has been shown to be high in the Finnish population, but linkage disequilibrium in general did not differ significantly from other populations (15); thus, our findings most likely cannot be explained by the particular population studied. Moreover, linkage disequilibrium between the CTLA4 polymorphisms has also been suggested in other studies (16, 17).

Due to linkage disequilibrium, it cannot be excluded that primary risk allele causing susceptibility to autoimmune diseases or even the observed effect on T cell activation, might also be some other variation near the CTLA4 gene or in some other closely linked gene. Among good candidates are the genes encoding for CD28 and ICOS that are located adjacent to CTLA4 and play important roles in the complex cascade of T cell activation (18).

In conclusion, although studies by Kouki et al. and Huang et al. indeed show important evidence for functional differences between CTLA4 gene variants, it is premature to conclude, in absence of data on all linked polymorphisms or without mutagenesis approach, which polymorphism is the primary disease allele in the CTLA4 gene region.

### References