Editorial Comment

HLA, Autoimmunity, and Rheumatic Heart Disease
Apparent or Real Associations?

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The report of Özkan et al in this issue of Circulation identifies HLA allelic associations with rheumatic heart disease (RHD) in a Turkish population, a population previously untested for HLA associations with RHD. Of interest, they report a significantly increased expression of B16, DR3, and DR7 in their patient sample. The increased expression of DR3 and DR7 alleles observed in their patients supports other reports of similar associations with RHD and adds to a growing overall compilation of purported class II associations with RHD including DR1, DR2, DR4, DRw6, and DQw2. Decreased expression among patients for DR5 was also found in their study; likewise, decreased allelic expression among patients for DRw6, DR5, DR8, and DR2 has been reported elsewhere.

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As a group, these independent studies implicate genes within or closely linked to the MHC complex as etiologically linked to RHD, but the dispersion of findings is unsettling and suggests heterogeneity in associations among different populations and/or nonspecificity in the methods of HLA antigen determination. Although discrepant, these data are nonetheless valuable in that collectively they implicate the MHC as a region containing genes that are associated with diseases with autoimmune features, such as RHD. These several studies also provide a point of departure for further investigations into etiological mechanisms (i.e., an interactive mechanism between streptococcal antigens and individual immune responses). However, it also is important to understand the limitations of serological microcytotoxicity typing methods in considering these study results.

Methodological Difficulties With Microcytotoxicity Typing

To understand the potential difficulties associated with serological typing, one must be aware of the complexity of the MHC region at both the genomic and phenotypic levels. Within the class II system, intact DR molecules consist of an α-chain and a β-chain, the latter being the most polymorphic and containing most of the serologically recognized differences. These variations are due to sequence differences in three variable regions in the amino-terminal domain of the β-chain. In addition, more than one serologically distinct β-chain can be expressed simultaneously in association with the α-chain. This overall complexity leads to overlapping "public" (shared) specificities along with specific, unique serological specificities among the different DR types. Moreover, within a serologically defined specificity, there usually are multiple allelic forms that cannot be detected serologically (Dw subtypes) and must be identified by other methods. Thus, HLA antigen typing established by serological testing may yield a broadly cross-reactive result on the one extreme and the inability to identify certain allelic differences at all on the other. Given this degree of variability in specificity, it is not difficult to understand why problems may arise in identifying disease associations with HLA types determined by serological microcytotoxicity testing.

Ambiguity in serological typing also can arise from the reagents used to assign HLA specificities. Typing sera are standardized by reactivity with cells of known HLA specificity. However, the actual epitopes recognized by individual sera usually are not known. The accuracy of typing is, therefore, a function of the quality and specificity of the typing sera, which may vary from batch to batch, and the extent to which the typing sera has been characterized. We have observed an example of an HLA-disease association, presumptively identified with a particular series of typing sera, that could not be confirmed when reagents from a different source were used (unpublished observations). These typing problems do not necessarily imply that HLA-disease associations are not present but do suggest that further characterization will be required to identify the linkage marker.

There can be other technical difficulties associated with HLA typing by microcytotoxicity. The basis of this test is that cells expressing a particular HLA specificity will incur membrane damage when treated with the corresponding typing serum and complement. The resulting loss of membrane permeability then is measured by the uptake of dye. The most common technical difficulty encountered is the variability of reactions with typing sera. This may result from poor viability of the cells used for typing, weakly reactive or cross-reactive sera, and presumably genetically determined differ-

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ences in the levels of cellular expression of these molecules. Because HLA-DR and -DQ specificities are only expressed on B cells, the method of isolation of this cell subset and the methods used to establish the purity of the resultant cell population are critical.

Potential Pitfalls With the Study Findings
Özkan et al. appear to be aware of some of these pitfalls, but it is unclear whether all of these concerns were addressed adequately. For example, they emphasized the importance of using multiple sera to confirm a specificity. This is unquestionably true; however, the 16 sera used to type the six HLA-DR specificities examined are significantly fewer than the 60–80 sera currently found on commercially available HLA-DR typing trays. Likewise, the DR specificities that they chose to examine have been further characterized into multiple “splits” of the original specificity. Failure to include these splits in the study eliminates about one half of the currently recognized HLA-DR specificities from consideration and leads to ambiguous associations with the “combined” or public specificities (e.g., DR3 has now been split into DRw17 and DRw18; is RHD associated with DRw17, DRw18, or both?). An even smaller proportion of the commonly recognized class I antigens were tested.

In certain individuals, two DR-β chains are expressed simultaneously. The products of the B3 and B4 genes encode β-chains that confer the HLA-DRw52 and HLA-DRw53 specificities. These specificities occur predictably in conjunction with certain DR alleles and can be used for confirmation of a DR assignment. Likewise, alleles from DR and DQ loci frequently show linkage due to their proximity. Common haplotype linkages can be of help in confirming or excluding questionable assignments. Özkan et al.1 did not include typing for DRw52-53 or DQ specificities, although they commented that more research must be done in this area.

In addition to technical problems, there are pitfalls associated with the experimental design that must be avoided. For studies of this sort in which multiple comparisons are made, there is a significant potential for type 1 error, that is, for a chance association between the presence of disease and a particular antigen, when, in fact, none exists. Özkan et al. recognized this problem and corrected the reported p values for multiple comparisons.11 It also is of paramount importance that the test and control samples be drawn from the same ethnic population (as was done) and that testing of these samples be done concurrently (not stated). In this way, differences among testing reagents are distributed evenly between the two sample groups, and artificial associations with the diseased group are avoided.

Thus, there are reservations to be mindful of in considering the findings of Özkan and coworkers. These arise largely from the inherent problems of the microtoxicity method, the relative paucity of the testing sera used, concerns regarding the method of choosing and testing controls, and the failure to take full advantage of known gene linkages in confirming assignments. This failure to include typing for all of the currently recognized specificities will necessitate extension and confirmation of the observations reported.

Beyond the inherent difficulties associated with microcytotoxicity, the many described associations between HLA alleles and various diseases generally suffer from a lack of prognostic usefulness in differing populations and in individual patients. Variability in the population associations can be reduced by limiting conclusions to populations that are well characterized genetically, but this also limits the general applicability of HLA typing. Despite the numerous associations that have been described, tissue typing generally has not proven to be a useful clinical diagnostic or prognostic tool in individual patients (with the exception of HLA-B27 in ankylosing spondylitis). This lack of applicability thus arises from the diversity of associations that have been reported for most of the diseases studied (including RHD) and the only modest predictive value of HLA typing for disease even in well-characterized populations.

Study Implications
The potential limitations described above do not render the findings of Özkan et al. uninteresting. Their work adds to accumulating evidence that implicates a role for the MHC in the pathogenesis of RHD and several other diseases with “autoimmune” features. Serological phenotype analysis continues to be the appropriate first step toward identifying associations of diseases with MHC.

As insight into the MHC is gained, a picture of increasing clarity emerges as to how these genes may influence the development of autoimmune disease. It is now dogma that T lymphocytes recognize foreign as well as self-peptides as a complex consisting of the peptide bound with the gene products of the MHC class I and class II loci.12 The array of peptides that can be presented to the immune system in this manner is dictated largely by the MHC alleles expressed by an individual. MHC polymorphism appears to have arisen as a phylogenetic attempt to acquire recognition capability for the vast universe of foreign antigens potentially encountered by an individual. In addition, other polymorphic genes have been linked to the MHC and encode proteins functionally important in the processing and presentation of immunogenic peptides. Several of these genes encode subunits of a proteolytic complex that degrades proteins into immunogenic peptides. Other MHC-linked genes encode transporter proteins that facilitate formation of the peptide–class I molecule complex.13 Thus, MHC gene products are crucial at several steps in the initiation of the immune response. Thus, as the functions of the molecules encoded within the MHC are clarified, pathoetiologic hypotheses linking HLA and disease should begin to emerge.

Future Directions
It is of interest to consider the future directions of investigations of HLA associations. The study of these HLA-disease associations is now possible on the molecular level. The 13 commonly identified DR antigens identified serologically have been expanded to 43 alleles based on identification by gene amplification (using the polymerase chain reaction) with typing by oligonucleotide hybridization.7 These developments have substantially improved our ability to specifically define and evaluate allelic differences. With these techniques in one study,14 for example, the recognized association...
between DR4 and insulin-dependent diabetes mellitus was found to correlate with only two of eight DR4 subtypes. These subtypes have been shown to differ in the amino acid sequence between positions 67–71 in the outer domain of the DRβ-chain. The fact that T lymphocytes can distinguish among DR subtypes has etiological implications and underscores the importance of examining DR alleles beyond their serological phenotypes.

Functional studies relating MHC genes to peptide processing and presentation are under way. HLA alleles have been shown to exhibit a hierarchy with regard to the peptide that is presented. The presence of a particular MHC allele may predispose to disease by causing exclusive presentation of a particular peptide to the immune system, leading to a specific pathological response. Consistent with this hypothesis, it recently has been demonstrated that an immunogenic peptide derived from myelin basic protein is recognized by cytotoxic T cells in the context of the four HLA DR types that have been found to be associated with multiple sclerosis. These new methodologies thus are rife with the potential for application in RHD and other autoimmune diseases.

The hypothesis that predisposing genetic factors are important in the development of RHD and other autoimmune diseases is not new, and the implications of HLA association with disease are uncertain. We are far from understanding fully the mechanisms through which an immune response to an individual's tissues may develop. However, our increasing knowledge of the function of the genes encoded within the MHC and of the mechanisms through which tolerance is achieved has given new life to the study of HLA and disease associations. The prospect for future advances is promising.

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