Clonal Rearrangements of Antigen Receptor Genes in the Diagnosis of Lymphoid Neoplasia (13-Nov-2004)

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Background Information
A clonal population of cells is defined as those cells arising from the mitotic division of a single somatic cell. The somatic mutation theory of carcinogenesis contends that a tumor results from the progeny of a single cell having acquired one or more somatic mutations [1]. Therefore, one of the most important features identifying a cell as being neoplastic is its clonal origin or derivation from a single cell [2].

In human pathology, the evaluation of clonality in lymphoid proliferations began approximately 20 years ago in people with the use of polyclonal antibodies directed against epitopes on immunoglobulin (Ig) light chains [3,4]. The identification of a significant population of lymphoid cells expressing a single light chain isotype, either κ or λ, was considered to indicate a monoclonal population because a mixture of κ- and λ-expressing cells would be expected in normal and reactive B cell populations [1,4]. This type of analysis proved useful in people for the assessment of both B cell chronic lymphocytic leukemia and B cell non-Hodgkin’s lymphoma [1,4]. In tissue sections, however, reliable detection of Ig light chain expression is often difficult to assess due to soluble Ig molecules overlaying the cellular Ig expression [5]. Additionally, the use of this type of clonality assessment is limited to B cell proliferations in humans and pigs. The utility of assessing light chain restriction is predicated upon the presence of an approximately balanced light chain ratio in health and it is therefore not useful in those species that have heavily skewed light chain expression patterns normally (dogs, cats, horses, cattle, sheep, chickens, mice and rabbits). There is no analogous system of surface markers indicating clonality in T cell tumors. Monoclonal antibodies specific for individual V regions or families of related V regions can act as indicators of the probable clonality of some T cell populations [6]. However, lack of a complete set of antibodies reduces the sensitivity of this technique and the specificity can be compromised in those situations where different clones utilize the same V region.

Indirect evidence of clonality can be obtained by identifying significant populations of T cells lacking expression of 1 or more pan-T cell antigens (or expressing an antigen not normally expected). This type of finding would suggest the presence of an abnormal clone of T lineage cells consistent with malignancy [7,8]. Similar conclusions can be drawn with the finding of an expanded population of immunoglobulin negative B cells [8]. However, this type of information is indirect evidence only and unequivocal confirmation of neoplasia is rarely possible solely by the use of immunophenotyping [9].

The more recent use of molecular probes to detect Ig and T cell receptor (TCR) gene rearrangement in lymphoproliferative disease has overcome many of the limitations of previous clonality assessment methods [1]. These techniques exploit the (physiologic) occurrence of molecular genetic rearrangements of the antigen receptor genes during T and B cell differentiation and are contingent upon knowledge of species specific antigen receptor DNA sequence information.

The Clinical Problem
Lymphoproliferative disease often presents the clinician and pathologist with a diagnostic dilemma, particularly early in the course of the disease. It can be very difficult to differentiate a reactive (polyclonal) lymphoid proliferation from a neoplastic (monoclonal) one and this distinction is a fundamental prerequisite for successful therapy and patient management. This
Molecular Clonality Diagnostics

When molecular clonality assessment studies were first undertaken in people, they successfully utilized Southern blot hybridization analysis [4,12,13]. There have also been two studies in dogs that have assessed clonality in lymphoproliferative disease utilizing southern blot molecular genetic analysis [14,15]. This technique is relatively sensitive and specific and while it is still recognized as the "gold standard", it suffers from many limitations [4,12,13,16]. Additionally, despite adequate sensitivity for detecting clonality, the technique lacks acceptable sensitivity for detection of minimal residual disease or early relapse. These factors markedly limit the utility of Southern blotting as a practical diagnostic methodology [12].

The problems associated with the use of Southern blot hybridization analysis for clonality assessment were circumvented in human pathology with the advent of the polymerase chain reaction (PCR) and its subsequent successful adaptation for the assessment of clonality in lymphoid proliferations [1,4]. The technique uses PCR to amplify the V(D)J splice junctions of the TCR or Ig gene segments in lymphocytes. The TCR and Ig gene loci contain many different V, D, and J gene segments which are subject to rearrangement processes during early lymphoid differentiation. The many different combinations of V, D, and J segments represent the so-called combinatorial repertoire. At the junction sites of the V, D, and J gene segments, further diversity is generated by the deletion and random insertion of nucleotides (N), via the enzyme TdT. The V-N-D-N-J junctional region is termed the complementarity determining region 3 (CDR3) and it is responsible for encoding much of the specificity of antigen binding of the respective receptor. The heterogeneity of N nucleotide addition (and deletion) between the V(D)J splice junctions produces a unique fingerprint for any given rearrangement that provides a sensitive and specific target for PCR amplification [17]. The products of a clonality PCR assay are resolved on a high resolution gel and visualized. Clonal or neoplastic proliferations produce 1 or 2 (due to rearrangement of both alleles) sharp bands on the gel while polyclonal or reactive proliferations produce a broad band or smear covering a range of product sizes [17]. In the study of clonality of T cell lymphoproliferative disease in humans, TCRG locus analysis provides a better diagnostic yield than TCRB locus analysis [18-22]. This is because the TCRG locus is more frequently rearranged in T cells, regardless of the surface TCR expression [19]. Additionally, TCRG locus rearrangements are generally easier to detect than TCRB locus rearrangements [13,23]. The limited number of V gene segments in the TCRG locus make it an easier system to select and optimize primers than the TCRB locus [13,23]. PCR based tests are rapid, exquisitely sensitive, applicable to very small quantities of DNA (including punch biopsies and fine needle aspirates) and can also be performed on formalin-fixed, paraffin-embedded tissue. These factors make it ideal as a basis for development of a reliable and convenient test for assessment of clonality. These factors also facilitate very sensitive detection of early relapse and minimal residual disease and retrospective studies on archival, formalin-fixed, paraffin-embedded material.

PCR based tests to assess clonality within lymphoid populations have been developed for the dog [9,24,25]. Similar testing has also been developed for the cat (Moore P.F et al., manuscripts submitted). Preliminary data at UC Davis assessing TCRG locus gene rearrangements found clonal TCRG gene rearrangement in 14/14 samples from dogs with TCRαβ expressing T cell CLL as well as 10/10 dogs with TCRγδ expressing T cell CLL. Therefore, similar to the situation in humans, TCRG locus analysis appears to provide a high diagnostic yield in the dog, regardless of surface TCR expression. DNA from normal dogs and dogs with inflammatory lymphocytoses resulted in the generation of a smear (as opposed to a clonal band) of correct expected size, suggesting that the specificity was also reasonable. A larger set of lymphoid malignancies in the dog (n=77), of both T and B cell phenotypes, was assessed by Burnett et al [24]. The sensitivity of the PCR assays in this study (the largest study to date in the dog) was approximately 90%, which is higher than the results of most human studies utilizing similar methods. There are several possible explanations for this. However, determination of the true sensitivity and specificity of these assays in dogs and cats will likely require assessment of a greater number of animals.

Limitations and Pitfalls

While molecular clonality studies can be highly informative, they are not without their limitations and pitfalls. False negative
results can occur for several reasons. Recognition of all possible V, (D), and J rearrangements is likely not possible with current primer sets in humans, dogs and cats. Although the primer sets in people are much more extensive than the current veterinary primer sets, they are still not complete [5]. The recent sequencing of the canine genome will likely assist in the refinement of the assay in dogs due to the potential development of greater coverage of all possible rearrangements. Secondly, the occurrence of somatic hypermutations in rearranged Immunoglobulin (Ig) genes of germinal center and post germinal center B-cell malignancies can result in subsequent false negatives due to improper primer annealing [5]. In people, this latter problem has been approached by designing additional "upstream" consensus primers to Framework 1 (FR1) and FR2 regions, complemented by concurrent use of primers that amplify light chain rearrangements and rearrangements of the kappa deleting element (Kde). Kde rearrangements are assumed to be free of somatic hypermutations and hence should represent more stable targets for PCR amplification [5].

False positive PCR results have been shown to be a serious problem in humans [5]. False positive results can occur if the analysis of obtained PCR products inadequately discriminates between monoclonal, oligoclonal or polyclonal PCR products. This is especially true when TCRG genes are used as PCR targets because of the more limited junctional diversity of TCRG rearrangements [5]. Because of this problem in human pathology, numerous analytical techniques have been utilized, including single-strand conformation polymorphism analysis, denaturing gradient gel electrophoresis, heteroduplex analysis and GeneScanning [13,16,26-29]. Most of these techniques resolve PCR products on the basis of both size and (unique) sequence. We have observed similar problems with occasional false positive results in samples known not to be neoplastic (W. Vernau and P.F. Moore, unpublished observations). Because of this, we routinely utilize heteroduplex analysis of our obtained clonality PCR products and this choice appears to have been validated by the recent report of the European BIOMED-2 collaborative study [5]. Pseudoclonality is another cause of false positive results that has been reported in humans [5,30,31]. We also have observed false positive pseudoclonal bands in the analysis of PCR clonality products in both dogs and cats (manuscripts submitted). Duplicate or triplicate PCR analyses run in parallel can assist in the detection of pseudoclonality [5] and we now routinely perform replicate analyses in dogs and cats (manuscripts submitted).

**Lineage Assignment**

Lineage assignment represents a further potential application for a PCR based clonality assay in animals. In humans, clonal analysis of antigen receptor genes has also been used to assist in the lineage assignment of lymphoid neoplasms, especially in those cases where immunophenotyping is non-diagnostic or ambiguous [32,33]. The finding of a TCR rearrangement was considered to justify definitive categorization of that lesion as being of T cell lymphoid lineage with comparable certainty for IgH rearrangements in B cell lymphoid neoplasms. While these conclusions generally hold true, they are not absolute or definitive when considered in isolation, and caution should be exercised when interpreting results for the purpose of lineage assignment [17]. Approximately 25 - 30% of high grade lymphoid tumors have "promiscuous" or cross-lineage rearrangements [34] (have rearrangements of both Ig and TCR genes). More than 90% of precursor B-ALL have been found to have cross-lineage TCR gene rearrangements [35] and 5 - 10% of well differentiated or low grade tumors have been found to have cross-lineage rearrangements too [18,36]. Additionally, antigen receptor rearrangement can occasionally be seen in acute non-lymphoid leukemias (ie. AML) which can also concurrently express some B cell associated antigens, further confounding interpretation [37-39]. We have observed similar cross-lineage rearrangements in dogs and cats and therefore rarely utilize gene rearrangement results for the purposes of lineage assignment.

**Summary and Caveats**

Sensitive and validated PCR based clonality assays in animals will serve many purposes. It should significantly improve our ability to provide an accurate diagnosis and prognosis for lymphoproliferative disease. It should expedite extremely sensitive detection of minimal residual disease and early relapse, thereby improving our ability to treat these diseases. Even greater sensitivity (10X - 1000X more sensitivity) is potentially achievable with the development and use of tumor specific or clonotypic primers [40,41]. Similar to the situation in people, PCR based clonality assays should be able to assist veterinary pathologists in achieving a level of diagnostic accuracy previously unattained for canine lymphoproliferative disease and should become an extremely valuable diagnostic and investigative adjunct.

Some final caveats are applicable to studies of clonality. While the demonstration of clonality is strongly suggestive of neoplasia, clonality alone does not prove the neoplastic theory or necessarily imply malignancy [4,42-44]. Monoclonal gammopathies have been described in association with confirmed inflammatory diseases in both people and dogs. Similarly, benign clonal expansions of T cells have been documented in people in association with some inflammatory diseases, acute viral disease or ageing. The presence of clonality in lymphoproliferative disease must be interpreted together with the clinical, morphologic and immunophenotypic findings [5,44]. These types of molecular genetic evaluations should be used as diagnostic adjuncts, not as replacements for more traditional methods of diagnosis. Accurate morphologic and immunophenotypic assessment must remain the cornerstone in the diagnosis of lymphoproliferative disorders [4]. Therefore,
optimal patient care should include the integration of immunophenotypic and clonality assessment with historical, clinical, morphologic and other (cytochemical, cyogenetic) information.

References

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