Antibody-Based Diagnostic Tests for Lyme Disease

Most diagnostic tests are based on the detection of specific antibodies in the blood of patients suspected of having a given infection. Such tests, especially those that have survived years of rigorous scrutiny and have been validated and approved for clinical use by the Food and Drug Administration (FDA) and the Centers for Disease Prevention and Control (CDC), have been invaluable in the control, diagnosis, and treatment of many infectious diseases. Notwithstanding, they have limitations that must be considered in evaluating the results obtained. In this presentation, these limitations will be considered with respect to the diagnosis of Lyme disease which historically has been defined as a tick-borne infectious disease caused by the spirochete, *Borrelia burgdorferi* (1).

Although sensitivity and specificity are decisive factors in judging the value of any antibody-based diagnostic test, several variables influence the assessment of each of these parameters. Of primary concern is the minimal amount of antibody that must be present, in a reasonable volume of blood or some other body fluid, to give a positive test result. Here, an understanding of the concept of "antigen load" with reference to the least amount of antigen required to stimulate the immune system to produce a detectable serum antibody response, is useful.

The term "antigen" refers to any substance that stimulates the immune system to make antibodies that are specific for that antigen. For *B. burgdorferi*, this consists of an array of several well-characterized molecules that are present on its cell surface in extremely small amounts. Most are proteins or lipoproteins that are characteristic of *B. burgdorferi* and play a role in pathogenesis (2); some are considered to have potential diagnostic significance, although others (e.g., the flagellar antigens) may be shared by unrelated nonpathogenic bacteria. Antibodies against shared or cross-reactive antigens such as flagella certainly are produced during Lyme disease; however, they are not diagnostically relevant since they are not produced only by *B. burgdorferi*, the causative agent of Lyme disease. Another major variable is the immunogenicity of these surface antigens, i.e., their inherent capacity to generate an antibody response. Some may be strongly immunogenic whereas others may be weakly immunogenic.

Aside from the issue of inherent immunogenicity, there is abundant evidence to indicate that the magnitude of an antibody response is dependent upon the amount of antigen available to stimulate the immune system. As the amount (dose) of antigen is increased (antigen load), more antibody is produced in a dose-dependent manner until a peak level is attained. It is relatively easy to determine the optimal dose required to generate a detectable antibody response against an isolated purified antigen or a vaccine, with or without the use of an adjuvant to enhance immunogenicity; however, it is much more difficult to do so when antigen is delivered in the form of live bacterial cells, and when the total amount delivered is dependent upon the extent to which bacterial cells multiply (replicate) during the course of infection.

In contrast to rapidly growing bacteria such as *Escherichia coli* or *Staphylococcus aureus* that replicate by cell division once every 17-30 minutes (3), *B. burgdorferi* replicates much more slowly -- once every 12-24 hours (4). Thus, although detectable amounts of antibody may be present in the blood within a few days after infection with rapidly growing pathogens, it may take several weeks before detectable amounts of antibody appear in the blood of patients infected with slow-growing *B. burgdorferi*. This, as well as the numbers of bacteria initially transmitted by an infected tick, influences the sensitivity of a

diagnostic test and is a major limitation as to when, during the course of an infection, a given antibody-based tests can be used for diagnostic purposes, regardless of specificity.

For someone living in an endemic area, the "bull's eye "or erythema migrans rash (EM) is considered to be sufficiently diagnostic for Lyme disease to justify antibiotic therapy, without the need for a positive serological test result (5). It develops at the site of a tick bite, within 7-14 days after an infected tick has taken a blood meal and detached (6, 7). Usually, patients with EM are seronegative at the time of presentation; however, the probability of becoming seropositive increases with the duration of EM and as infection becomes more disseminated, i.e., as antigen load increases, and the amount of antibody produced reaches detectable levels in the blood (8, 9, 10). Although only 25-50% of patients with EM are ELISA positive during the early or acute phase of their infection, 80-90% of treated EM patients are seropositive as the duration of the EM is increased and by convalescence. This occurs, despite the fact that such patients were treated with an antibiotic that may have reduced "antigen load" to some degree, thereby lessening the magnitude of the resultant antibody response (10, 11).

As one would anticipate, and has been elegantly demonstrated by several investigators, the sensitivity of an antibody-based diagnostic test for Lyme disease increases progressively with the duration of EM (10), and in patients with: (a) acute neurologic or cardiac abnormalities; and, with (b) arthritis or chronic neurologic abnormalities (11). Under such circumstances, and as infection becomes more disseminated, both sensitivity and specificity range from 85% to 99% (11). Similar results were obtained when the same specimens were assayed in parallel to two-tier testing by another highly specific antibody-based procedure, the Vlse C6 peptide ELISA (10, 11).

Because of the acknowledged low sensitivity of ELISA tests during the early acute EM phase, neither the CDC nor the Infectious Diseases Society of America (IDSA) advocate serological testing of such patients. Rather, they consider it appropriate to treat such patients with antibiotics, and then do follow-up serological testing during convalescence, when detectable amounts of antibody are likely to be present in blood (4). Thus, the early or acute EM phase of Lyme disease is the only time during active infection when the sensitivity of two-tier testing is low.

Despite the above mentioned well-documented observations, some continue to discount the validity of two-tier testing as a diagnostic for Lyme disease, claiming that its sensitivity is "no better than that of a coin toss" (12). However, those who hold such a view ignore the fact that most -- if not all -- of the test results upon which their argument is based were derived from patients with EM, i.e., from patients with early acute Lyme disease when the immune response is just beginning and antibody levels are very low (12). Obviously, under such circumstances, low sensitivity is surely to be expected as has been reported by other investigators (8, 9, and 10); this is why testing is not recommended under such circumstances (5). Specificity is not an issue since whenever antibody is detected -- albeit in small amounts -- it is found to be 99-100% specific for *B. burgdorferi* (12).

It should be noted – and emphasized—that the criteria currently recommended by the CDC for the interpretation of IgM and IgG Western blots for the diagnosis of Lyme disease were not selected arbitrarily, but are based on objective statistical considerations. This included an analysis of the frequency of specific bands detected in well-characterized specimens obtained from cohorts of patients with Lyme disease, in contrast to appropriate controls. The analysis also involved calculating the receiver operating characteristic (ROC) areas for each of the most common IgM and IgG bands observed. In a retrospective analysis of 225 case and control subjects (13), the best discriminatory ability of test

criteria, i.e., one that would yield the greatest incidence of unequivocal positive values in combination with the lowest incidence of false positive values, was obtained by requiring at least 2 of the 8 most common IgM bands detected in early disease (18,21,28,37,41,45,58,and 93kDa) and by requiring at least 5 of the 10 most frequent IgG bands detected after the first weeks of infection (18,21,28,30,39,41,45,58,66,and 93kDA). These criteria are those currently used and recommended by the CDC for the 2-tier serologic diagnosis of Lyme disease, although it should be noted that more recent advancements in technology have resulted in significant improvements in sensitivity and specificity (16).

Note that bands 31 (OspA) and 34 (OspB) are not included in the abovementioned CDC criteria for 2-tier testing. That is because these bands were found to be present in low frequency – if at all—in patients with either arthritis or late neuroborreliosis (13). It was discovered subsequently that, although OspA and OspB are expressed by Borrelia when cultivated on artificial laboratory media or in the mid-gut of infected ticks, these surface antigens are not usually expressed in mammalian hosts during the course of infection (14, 15). Consequently, antibodies specific for OspA and OspB are not considered to be of value for the diagnosis of Lyme disease. This justified the use of LYMErix as an OspA-based, transmission-blocking vaccine for the prevention of Lyme disease, without compromising the diagnostic value of 2-tier testing.

The dissemination of misleading information on the validity of two-tier testing (12) at best reflects ignorance of both the disease process and limitations associated with antibody-based tests when used for the diagnosis of Lyme disease. At worse, it is an inept attempt to selectively report only those observations that serve to discredit the validity of two-tiered testing. Neither is in the best interest of the public health or helpful to patients who suspect that they may have Lyme disease.

There is abundant evidence indicating that two-tier testing has performed well when applied under conditions when the probable risk of contracting Lyme disease is reasonably high (17, 18). Those competent and experienced in the use of diagnostic tests understand that the results obtained with antibody-based diagnostic tests are valid only when such tests are used when detectable amounts of antibody are likely to be present. Even the most sensitive and specific diagnostic test one can imagine is not going to indicate that a patient has Lyme disease, if that patient doesn't have Lyme disease in the first place. Alternatively, if a patient has long-standing non-specific symptoms of a type often associated with Lyme disease but is seronegative, it is not prudent to treat such patients with an extended course of antibiotics for an infection that may not even exist. It makes more sense to consider other causes for their symptoms (see: http://www.aldf.com/wp-content/themes/ALDF/pdf/The_Pain_of_Chronic_Lyme_Disease_FASEB_full_article.pdf).

Certainly, continual efforts should be made to improve existing technology so that diagnostic tests are able to detect small amounts of antibody during the course of an infection; this would ensure that curative antibiotic therapy can be commenced as early as possible. This is now being done. The recent establishment of a reference serum repository in which the results of newly developed diagnostic tests can be compared to those derived from existing procedures -- using identical well-characterized panels of reference specimens in both cases-- will greatly accelerate progress in that regard (19).

The ELISA format, with a sensitivity estimated to range from 0.01 to 0.1 nanograms of antibody per milliliter (20), seems to be ideally suited for early diagnostic testing. Its remarkable sensitivity is due largely to the ability of an enzyme conjugated to a second antibody to amplify the reaction between a single specific antibody molecule and its relevant ligand (or antigen), by a factor of 10,000-fold or more.

The replacement of bacterial cell lysates often used in conventional ELISAs with well-defined peptides associated with specific antigens produced early in infection (21) reduces variability and greatly facilitates comparisons of results from different laboratories. The Vlse C6 peptide ELISA is but one example of just such an application. Parallel testing has shown that it gives results comparable to those obtained with the conventional ELISA used in the standard two-tier test procedure. However, it should be noted, that there have been no reports of patients who are seropositive for Lyme disease by the Vlse C6 peptide ELISA, who are not also seropositive by the conventional ELISA (10, 11). Although sensitivity appears to be equivalent since the results obtained have been comparable in all instances (10, 11), additional comparative studies are needed before this single diagnostic test can replace two-tier testing.

In recent studies using the Luminex multiplex assay system, 10 markers were selected from an initial analysis of 62 *B. burgdorferi* surface proteins and synthetic peptides by assessing binding of IgG and IgM antibody to each in a training set of Lyme disease patients samples and controls (22). In a validation set, this 10-antigen panel identified a higher proportion of early Lyme disease patients as positive at the baseline or post-treatment visit compared to two-tiered testing (87.5% and 67.5%, respectively, $p<0.05$). Equivalent specificities of 100% were observed in comparisons to 26 healthy controls. Upon further analysis, positivity with respect to the novel 10-antigen panel was associated with longer illness duration and multiple EM rash. The improved sensitivity and comparable specificity of the 10-antigen panel compared to two-tiered testing in detecting early *B. burgdorferi* indicates that multiplex analysis, featuring this next generation of new markers, could advance diagnostic technology significantly, resulting in better diagnosis and treatment of early Lyme disease.

It should be noted that the procedure recommended by both the Centers for Communicable Disease Control (CDC) and the Food and Drug Administration (FDA) for the diagnosis of Lyme disease is a two-step procedure. It includes an ELISA test which, if positive for Lyme disease, is confirmed by either an IgM or IgG Western blot; since an IgM Western blot is reliable only during the very early stages of infection, an IgG Western blot is recommended for infections of 4 or more months in duration. Because the Western blot is not a quantitative procedure, it is only able to detect the presence of antibody specific for *Borrelia*, not the amount of such antibody. Consequently, a Western blot -- as usually performed -- is unable to distinguish between the relatively large amounts of different types antibodies produced during active infection and the smaller amounts of those same antibodies that might be present for as long as 10-20 years after patients have been given recommended antibiotic therapy, i.e., background antibodies (23). Obviously, in the absence of direct evidence of active infection, it makes no sense to treat with antibiotics until a patient becomes seronegative – or until symptoms attributed to Lyme disease disappear. Alternative possibilities should be considered (see: http://www.aldf.com/wp-content/themes/ALDF/pdf/The_Pain_of_Chronic_Lyme_Disease_FASEB_full_article.pdf).

Although the basis for the persistence of such background antibody is not known, it may be the result of prolonged antigenic stimulation. Since *Borrelia* are known to produce several different integrins or adhesion molecules that enable them to bind tenaciously to various mammalian cells (24), the ability of intact dead *Borrelia* -- or their remnants-- to persist in mammalian tissues for long periods of time after successful antibiotic treatment and cure might be sufficient to stimulate the immune system to produce detectable amounts of antibodies for many months to 10-20 years after the infection has been cured by antibiotic therapy (25,26). It also should be noted that these same adherent intact dead *Borrelia* or their remnants have been shown to possess pharmacologically active lipoproteins that could contribute to the expression of local persistent symptoms after the infection has been cured by antibiotic therapy

(27). In this context, it has been reported that the number of arthritic episodes in patients with antibiotic refractory Lyme-induced arthritis declines at a progressive rate (about 20% per year) until they no longer occur, 9 years after the infection was cured initially by antibiotics, in the absence of further antibiotic therapy (28).

Obviously, quantitative ELISAs used in conjunction with well-defined ligand specific for *Borrelia* would be vastly superior to Western blots to assess the efficacy of antibiotic therapy, as well as distinguishing between high levels of antibody characteristic of active infection and low levels of background antibodies that might be found in some patients who have been successfully treated for their infection and cured. The time is long past due to move the diagnosis of Lyme disease in that direction. The establishment of a highly recommended Lyme disease reference specimen repository by the CDC, in collaboration with the NIH and FDA, should facilitate progress in that regard (http://www.aldf.com/2nd-banbury-conference/).

In view of the above-mentioned limitations of existing diagnostic procedures to detect early Lyme disease, novel approaches, e.g., metabolomics, are being explored to detect and validate the detection of individual molecules or panels of molecules that can be used as biomarkers or signatures of specific infection, very early during the course of a disease. One such study demonstrates a unique metabolic biosignature with a sensitivity of 84%-95% and a specificity of 90%-100% that might be used for the detection of early Lyme disease (29). This approach also was found to be useful in differentiating early Lyme disease from southern tick-associated rash illness or STARI (30). Obviously, additional comparative studies are needed to more fully assess the feasibility of this approach for the routine diagnosis of Lyme disease.

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