Understanding the Western Blot

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Inquiries about various issues relating to Western blot (WB) testing are frequently posted to the Lyme disease discussion groups on the Internet. Among the most commonly asked questions are: What laboratory techniques are used to carry out the assay? What exactly is being measured? What is a "band"? How are the results interpreted? What are the CDC criteria for a "positive" test? Although some of the medical jargon associated with immunology can be a little overwhelming, the scientific principles behind these tests are not difficult to grasp. The following article is offered as a primer in the techniques and interpretation of Western blotting, and should help most patients navigate their way through some of the medical and scientific terminology associated with the assay.

First of all, it should be noted that the Western blot is usually performed as a follow-up to an ELISA test, which is the most commonly employed initial test for Lyme disease. "ELISA" is an acronym for "enzyme-linked immunosorbent assay." There are ELISA tests and Western blots for many infectious agents; for example, the usual testing regime for HIV is also an initial ELISA followed by a confirmatory Western blot.

Both the ELISA and the Western blot are "indirect" tests -- that is, they measure the immune system's response to an infectious agent rather than looking for components of the agent itself. In a Lyme disease ELISA, antigens (proteins that evoke an immune response in humans) from *Borrelia burgdorferi* (Bb) are fixed to a solid-phase medium and incubated with diluted preparations of the patient's serum. If antibodies to the organism are present in the patient's blood, they will bind to the antigen. These bound antibodies can then be detected when a second solution, which contains antibodies to human antibodies, is added to the preparation. Linked to these second antibodies is an enzyme which changes color when a certain chemical is added to the mix. Although the methodology is somewhat complicated, the basic principle is simple: the test looks for antibodies in the patient's serum that react to the antigens present in *Borrelia burgdorferi*. If such antibodies exist in the patient's blood, that is an indication that the patient has been previously exposed to *B. burgdorferi*.

Cross-reacting antibodies

However, many different species of bacteria can share common proteins. Most Lyme disease ELISAs use sonicated whole *Borrelia burgdorferi* -- that is, they take a bunch of *B. burgdorferi* cells and break them down with high frequency sound waves, then use the resulting smear as the antigen in the test. It is possible that a given patient serum can react with the *B. burgdorferi* preparation even if the patient hasn't been exposed to Bb, perhaps because Bb shares proteins with another infectious agent that the patient's immune system *has* encountered. For example, some patients with periodontal disease, which is sometimes associated with an oral spirochete, might test positive on a Lyme ELISA, because their sera will react to components of Bb (like the flagellar protein, which is shared by many spirochetes) even

though they themselves have never been infected with Bb. Therefore, some positive Lyme disease ELISA results can be "false" positives.

To distinguish the false positives from the true positives, a more specific laboratory technique, known as immunoblotting, is used. (The Western blot, which identifies specific antibody proteins, is but one kind of immunoblot; there is also a Northern blot, which separates and identifies RNA fragments, and a Southern blot, which does the same for DNA sequences.) In a Western blot, the testing laboratory looks for antibodies directed against a wide range of Bb proteins. This is done by first disrupting Bb cells with an electrical current and then "blotting" the separated proteins onto a paper or nylon sheet. The current causes the proteins to separate according to their particle weights, measured in kilodaltons (kDa). From here on, the procedure is similar to the ELISA -- the various Bb antigens are exposed to the patient's serum, and reactivity is measured the same way (by linking an enzyme to a second antibody that reacts to the human antibodies). If the patient has antibody to a specific Bb protein, a "band" will form at a specific place on the immunoblot. For example, if a patient has antibody directed against outer surface protein A (OspA) of Bb, there will be a WB band at 31 kDa. By looking at the band pattern of patient's WB results, the lab can determine if the patient's immune response is specific for Bb.

Here's where all the problems come in. Until recently, there has never been an agreed-upon standard for what constitutes a positive WB. Different laboratories have used different antigen preparations (say, different strains of Bb) to run the test and have also interpreted results differently. Some required a certain number of bands to constitute a positive result, others might require more or fewer. Some felt that certain bands should be given more priority than others. In late 1994, the Centers for Disease Control and Prevention (CDC) convened a meeting in Dearborn, Michigan, [1] in an attempt to get everybody on the same page, so that there would be some consistency from lab to lab in the methodology and reporting of Western blot results.

IgG and IgM

Before we get to the recommendations that resulted from this meeting, we need to understand one more facet of the human immune response. Many patients have noticed that their Western blot report is actually comprised of two separate parts, IgM and IgG. These are immunoglobulins (antibody proteins) produced by the immune system to fight infection. IgM is produced fairly early in the course of an infection, while IgG response comes later. Some patients might already have an IgM response at the time of the EM rash; IgG response, according to the traditional model, tends to start several weeks after infection and peak months or even years later. In some patients, the IgM response can remain elevated; in others it might decline, regardless of whether or not treatment is successful. Similarly, IgG response can remain strong or decline with time, again regardless of treatment. Most WB results report separate IgM and IgG band patterns and the criteria for a positive result are different for the two immunoglobulins.

Finally, in setting up a nationwide standard for a positive WB, one makes several assumptions -- that all strains of Bb will provoke similar immune responses in all patients, that all patients will mount a measurable immune response when exposed to Bb, and that the IgG immune response will persist in an infected patient. Unfortunately, none of these is always true. Therefore, a judicious interpretation of Western blot results in a clinical setting

should take into account both the vagaries of the human immune response and the possibility that strain variations in Bb might produce unusual banding patterns.

Official criteria

The CDC criteria for a positive WB are as follows:

* For IgM, 2 of the following three bands: OspC (21-25), 39 and 41. * For IgG, 5 of the following ten bands: 18, OspC (21-25), 28, 30, 39, 41, 45, 58, 66 and 93.

How were these recommendations arrived at? The IgG criteria were taken pretty much unchanged from a 1993 paper by Dressler, Whalen, Reinhardt and Steere [2]. In this study, the authors performed immunoblots on several dozen patients with well characterized Lyme disease and a strong antibody response and looked at the resulting blot patterns. By doing some fairly involved statistical analysis, they could determine which bands showed up most often and which best distinguished LD patients from control subjects who did not have LD. They found that by requiring 5 of the 10 bands listed, they could make the results the most specific, in their view, without sacrificing too much sensitivity. ("Sensitivity" means the ability of the test to detect patients who have the disease, "specificity" means the ability of the test to exclude those who don't. Usually, an increase in one of these measures means a decrease in the other.)

The IgM criteria were determined in much the same fashion (by different authors in different papers). Fewer bands are required here because the immune response is less mature at this point. Several studies have shown that the first band to show up on a Lyme disease patient's IgM blot is usually the one at 41 kDa, followed by the OspC band and/or the one at 39. The OspC and 39 kDa band are highly specific for Bb, while the 41 kDa band isn't. That's why the 41 by itself isn't considered adequate. Here's the rub, though: the CDC doesn't want the IgM criteria being used for any patient that has been sick for more than a month or two. The thinking here is that by this time an IgG response should have kicked in and the IgM criteria, because they require fewer bands, are not appropriate for patients with later disease.

Criticism of CDC criteria

A number of criticisms have been offered of the CDC criteria since their adoption in 1994. The first is centered on the CDC's failure to make any qualitative distinction among the various bands that can show up on a patient's Western blot. A number of Lyme disease researchers feel that different bands on a WB have different relative importance -- that "all bands are not created equal." For example, many patients with Lyme disease will show reactive bands at, say, 60 and/or 66 kDa. However, these correspond to common proteins in many bacteria, not just Borrelia burgdorferi, and so are of limited diagnostic usefulness, especially in the absence of other, more species-specific bands. The band at 41 kDa corresponds to Bb's flagella (the whip like organelles used for locomotion -- Bb has several) and is one of the earliest to show up on the Western blots of Lyme disease patients. But for some reason it is also the most commonly appearing band in control subjects. This may be due to the fact that many people are exposed to spirochetes at some time in their lives and so their sera might cross react with this protein.

On the other hand, certain other bands are considered highly specific for Bb -- the aforementioned 31 kDa band, for example, or 34 (OspB) or 39 or OspC (anywhere between

21 and 25). The 83 and 94 kDa bands are also thought to be species-specific. Many Lyme disease scientists believe that any patient whose IgG Western blot exhibits bands at, say, any three (or even two) of these locations almost certainly has Lyme disease, regardless of whether or not any other bands are present. They feel that these bands on a Lyme Western blot are simply more meaningful than other, less specific ones and that a rational interpretation of a WB result should take this into account. Unfortunately, this does not often happen, and will happen even less with the new CDC criteria [*Ed. Note: This paper was written in 1996. The criteria have remained the same.*]

A second criticism of the CDC Western blot criteria is that they fail to include the 31 and 34 kDa bands. This does indeed seem like an odd decision, since antibodies with these molecular weights correspond to the OspA and OspB proteins of B. burgdorferi, which are considered to be among the most species-specific proteins of the organism. So why didn't Dressler et al. include them? Answer: These bands tend to appear late if at all in Lyme disease patients, and did not show up with great frequency in the patients that the Dressler et al. group studied (though they did show up sometimes). As a result, they weren't deemed to have much diagnostic value and didn't find their way onto the CDC hot list. However, while the absence of either of these bands from a patient's immunoblot result does not rule out Lyme disease, their presence is hardly meaningless. Thus, many Lyme disease experts believe it is a serious mistake to exclude these two antibody proteins from the list of significant bands. The CDC's decision to do so seems particularly strange in light of the fact that it is the OspA component of Bb that is being used as the stimulating antigen in the ongoing experimental Lyme disease vaccine trials. As one immunologist remarked shortly after the 1994 CDC conference, "If OspA is so unimportant, then why the heck are we vaccinating people with it?"

False negatives

Finally, it is important to keep in mind that no matter how carefully the Western blot test is carried out and interpreted, its usefulness, like that of all tests that measure *B. burgdorferi* antibodies, is ultimately contingent on the reliability of the human immune response as an indicator of exposure to *B. burgdorferi*. There are several scenarios in which the lack of a detectable antibody response may falsely point to a lack of *B. burgdorferi* infection. First, it is well established that early subcurative treatment of Lyme disease can abrogate the human immune response to *B. burgdorferi* [3]. Although this is not thought to be a common phenomenon, a recent comparative trial for the treatment of erythema migrans found that a majority of patients who failed early treatment and suffered clinical relapse were seronegative at the time of relapse [4]. Even treatment for disseminated Lyme disease, in which the patient's IgG immune response was previously well-established, can render a patient seronegative after treatment despite post-treatment culture-positivity for *B. burgdorferi* [5,6].

In addition, patients with Lyme disease may not test positive for exposure to *B. burgdorferi* because their antibodies to the organism are bound up in immune complexes [7]. Once steps are taken to dissociate these immune complexes, free antibody can be detected; however, this is not routinely done when performing serologic tests for Lyme disease. Finally, an indeterminate number of patients with late Lyme disease are simply seronegative for unknown reasons [8]. The actual percentage of such cases as a proportion of all Lyme disease cases is impossible to estimate, since most studies of late Lyme disease enroll only

seropositive patients, which tends to reinforce the circular and erroneous notion that virtually all patients with late Lyme disease are seropositive.

It should also be noted that a positive Western blot is not necessarily an indication of active Lyme disease. A patient's immune response to *B. burgdorferi* can remain intact long after curative treatment for a Lyme infection; therefore, the results of a Western blot assay should always be interpreted in the context of the total clinical picture.

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References

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