Lyme disease (LD) is difficult to diagnose absent hallmark clinical symptoms. LD immunoassays are problematic because of cross-reactivities, false-positives, sequestration of antibody and antigen in immune complexes (IC) early after infection, and persisting antibodies. Demonstration of IC in LD (10) opened the possibility of developing assays to detect immunoglobulin M (IgM) in IC at a time when antibiotic therapy would be useful. Subsequently, we confirmed the presence or absence of LD antigens/antibodies in sera and other biological fluids from putative LD patients (1–4). Assay development protocols (antibody/antigen checker-boards to ascertain correct concentrations of primary and secondary antibodies), appropriate blocking reagents, and sensitive detection methods (e.g., biotin-avidin), we obtained superior sensitivity and specificity of IC over comparator tests (3, 4). We compared our enzyme-linked immunosorbent assay and immunoblots with many types of commercial (e.g., MarDx) and laboratory (e.g., immunofluorescent antibody, enzyme immunoassays) tests. Using monoclonal antibodies (MAb), standard assay development protocols (antibody/antigen checker-boards to ascertain correct concentrations of primary and secondary antibodies), appropriate blocking reagents, and sensitive detection methods (e.g., biotin-avidin), we obtained superior sensitivity and specificity of IC over comparator tests (3, 4) or whole serum alone (corroborated in independent laboratories). IC allowed us to “see” more bands in immunoblots (2) than a comparator commercial test, and exhaustive absorption of IC along with a “good” MAb even allowed us to detect Borrelia burgdorferi antigen in early infection (1, 2).

Although a report by Marques et al. (9) refutes beneficial use of IC in LD assays, there are enormous differences between their work and mine.

To emulate our protocols (1, 2), Marques et al. (9) measured total IgM and IgG in IC (and sera) by first concentrating IC at 2:1 after the polyethylene glycol (PEG) precipitation step and then making dilutions (IC, 1:10; sera, 1:100) and measuring them by nephelometry. After correcting for concentration and dilution, they arrived at an IC having 3.2-fold more IgM (and 50% less IgG) than unprocessed sera. In the Discussion, they attribute the larger amount of immunoglobulin in IC to extra immunoglobulin contributed by antibody present in unprocessed sera adding to that contributed by IC, to justify their experimental finding (9). More likely, “overmanipulation” of the IC sample to be measured (first concentrating—for no obvious reason—then diluting and measuring the final dilution by nephelometry) is the probable cause for the inconsistent measurement. They confused my protocol (which used a 2:1 concentration) with the one I used for antigen detection (1, 2), where enrichment of IC (by volume reduction before adsorption with beads) may be important for antigen detection.

Recognizing the importance of total IgM in IC and whole sera (2) in early infections, we developed a quantitative IgM assay. We coated plates with an excess (10 μg/ml) of anti-IgM that reacted with dilutions of an IgM standard (whole-molecule human IgM myeloma; Jackson ImmunoResearch), compared to dilutions of unknowns (IC, not previously concentrated, and unprocessed sera), and calculated the values of unknowns that fell in the linear portion (50 to 500 ng/ml) of the standard curve by linear regression (using anti-human IgM–horseradish peroxidase second antibody reporter), as in typical protein assays. There was a range of IgM concentrations three- to sevenfold greater in sera than IC for all LD and non-LD sample pairs tested. This was confirmed independently by nephelometry (SmithKline Beecham). These values were used to normalize total IgM in sera and IC and demonstrated superior sensitivity of IC for detecting LD patient antibodies in immunoblots (see Fig. 2c in reference 2). This was a more rigorous method for measurement. Even if some free antibody was trapped in their PEG precipitate (as they claim in their Discussion to account for their result of higher immunoglobulin content in IC), the validity of their measurement seems unlikely. Unless PEG precipitation somehow adds IgM, their result violates conservation of mass. Digeon et al. have already shown only negligible amounts of free antibody are precipitated with 3.5% PEG (5).

An even greater objection is to their attempts to detect antigen in IC (9). They used a 1:10 dilution of monoclonal antibody H5332 supernatant obtained from Alan Barbour. They first tried a semidry transfer with a sensitive chemiluminescent reagent and got multiple bands, including the 31-kDa band, for both patients and controls. They state that using several types (e.g., GammaBind-G-Sepharose, mannan–binding protein, protein–agarose) of antibody binding beads “failed to remove the nonspecific binding.” That is not the intended purpose of using these beads, which simply react with the Fc portion of antibodies of their respective isotype. They lowered the H5332 concentration to 1:100, which did not remove nonspecificity. They dissociated the IC with acid of pH 3.5 (possibly detrimental to the complex, which is why we used a high pH, 10.2). They tried a less sensitive chemiluminescent reagent with no success. Finally they used the more sensitive wet transfer with the less sensitive colorimetric detection, whereupon they obtained no bands. The main problem is their weak H5332 supernatant, which was either freeze-thawed, poorly stored, overpassaged, or otherwise improperly handled, and/or possibly incorrect dilution of their secondary antibody. They changed multiple variables (reagents and methods) simultaneously. They neglected to troubleshoot nonspecific bands with an unrelated MAb. They showed no immunoblots.

We purchased the original H5332 hybridoma cells from Alan Barbour in the late 1980s. We propagated these ourselves, using low-passage fresh supernatant (never frozen) at a 1:1,000 dilution (compared to their 1:10 dilution). The secondary antibody, horseradish peroxidase–goat anti-mouse IgG Fab specific (A-2304; Sigma), was used at a 1:150,000 dilution, along with Renaissance-plus (NEN) chemiluminescent reagent (no longer available). This gave one specific band at 31 kDa in Lyme disease patients and no bands in controls. The technique was used on 24 samples (patients of various LD manifestations and some controls) in one report (2), and one seronegative patient in another (1), and we showed immunoblots.

The techniques and materials used by Marques et al. are clearly so different from those I developed that there can be no direct comparison between their results and ours. Further, the authors may have been unaware of several studies where pathogen-specific antibody has been found in IC in infectious diseases prior to LD (5–8, 11).

Attempts to disprove the concept of IC by merely claiming
to repeat a procedure with poor materials, techniques, and multiple simultaneous methodological changes should be discounted. Their conclusions are invalid, counterproductive, and a disservice to patients with or suspected of LD.

REFERENCES


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Authors’ Reply

This is in regard to a letter to Dr. Michael Brunner in which he questions a recent paper of ours entitled “Detection of Immune Complexes Is Not Independent of Detection of Antibodies in Lyme Disease Patients and Does Not Confirm Active Infection by Borrelia burgdorferi” (1). Our main objective in the study was to evaluate the presence of antibodies in immune complexes using the PEG-IC method as a marker of active infection in patients with Lyme disease, not as a diagnostic tool. We have made no claims regarding the putatively increased sensitivity of IC-based methods when these are compared with other methods for the diagnosis of patients with early Lyme disease. As we discussed in the paper, our data actually would be in agreement with this possibility, but we offer for it an alternative explanation. The increased sensitivity is likely based not on “seeing” more bands in immunoblots but on the fact that the method used for the preparation of the PEG-IC does precipitate not only immune-complexed antibody, as argued by Dr. Brunner, but also free antibody.

Regarding Dr. Brunner’s objections on the validity of our attempts to detect antigen in the IC, these were our results after trying all of the improvements and alternatives to the method that were feasible and following the protocols of both Dr. Brunner and Dr. Schutzer as closely as possible. While Dr. Brunner claims that our inability to detect complexed OspA was due to a “weak” antibody (H5332), he fails to mention that we actually used not just one but three monoclonal antibodies against OspA, (H5332 from Dr. Barbour, C6550M from BioDesign, and MAB302 from Maine Biotechnology) as well as a polyclonal anti-Borrelia burgdorferi antibody (B65304P; BioDesign). All of the monoclonal antibodies yielded a single band using a Borrelia whole-cell lysate as antigen on immunoblots and reacted with purified OspA, which was used as control in all the blots. Dr. Brunner claims that we changed multiple variables simultaneously and that we neglected to troubleshoot nonspecific bands. Both of these assertions are not correct, as described by him in the letter and in our paper; Dr. Brunner also omits to mention that we actually consulted with him on multiple occasions during our study, and several modifications were made following his recommendations, including some not described in the paper. Examples of Dr. Brunner’s recommendations included omitting washes at certain points, adding the mannan-binding protein beads, replacing our secondary antibody, reducing the level of Tween 20 used during blocking, trying the Western Lightning chemiluminescence reagent, modifying the sample reducing buffer, and attempting the colorimetric protocol. These and other modifications were attempted over a two-and-a-half-year period, most with one modification made at a time. We did not show immunoblots in the paper, as we did not think it would be helpful, but Dr. Brunner actually has seen some of the blots. Simply put, we made a very significant effort to try to replicate the immunoblot results of Dr. Brunner that showed the presence of B. burgdorferi antigens in immune complexes but were not able to do so.

Dr. Brunner is correct in stating that our results on the quantification of total IgG and IgM in the PEG-IC are too high. In reviewing the data, we realized that a mistake was made in the calculation (a pertinent Authors’ Correction has been submitted): the concentrations of IgG and IgM in immune complexes were erroneously multiplied by a factor of 6 and therefore overreported by this factor. Therefore, the IgG concentration in immune complexes was 93 mg/dl, not 558.5 mg/dl, and that of IgM was 86.5 mg/dl, not 519 mg/dl. As reported, the IgG and IgM concentrations in serum were 1,030 mg/dl and 163 mg/dl, respectively. Hence, the IgM values in the PEG-ICs were reduced by a factor of almost 2 with respect to serum, not increased as we had reported, and that of IgG was diminished 11-fold, and not just by a factor of 2. This error does not invalidate the main conclusion of our study, which is that the results of the B. burgdorferi ELISA using the PEG-IC preparation are not independent from the results using unprocessed serum and are not more likely to reflect active infection, and this is probably due to precipitation of not only immune-complexed but also free antibody during the PEG-IC preparation.

REFERENCES

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