

Detection of Immune Complexes Is Not Independent of Detection of Antibodies in Lyme Disease Patients and Does Not Confirm Active Infection with *Borrelia burgdorferi*

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The *Borrelia burgdorferi*-specific immune complex (IC) test, which uses polyethylene glycol (PEG) precipitation to isolate ICs from serum, has been used as a research test in the laboratory diagnosis of early Lyme disease (LD) and has been proposed as a marker of active infection. We examined whether *B. burgdorferi*-specific antibodies were present within PEG-precipitated ICs (PEG-ICs) in patients with LD, posttreatment Lyme disease syndrome, and controls, including individuals who received the outer surface protein A (OspA) vaccine. Using a *B. burgdorferi* whole-cell enzyme-linked immunosorbent assay (ELISA), we obtained positive PEG-IC results not only in patients with a history of LD, but also in individuals vaccinated with OspA vaccine. The frequency of positive PEG-IC ELISAs in OspA vaccinees was significantly higher with ELISA-reactive than with ELISA-negative unprocessed serum samples ($P = 0.001$), demonstrating dependency between the tests. Similar results were found using samples from rhesus macaques infected with *B. burgdorferi*, uninfected macaques vaccinated with OspA, and controls. Therefore, testing for the presence of antibodies against *B. burgdorferi* in PEG-IC preparations is not more likely to reflect active infection than testing in unprocessed serum and should not be used in individuals who received the OspA vaccine.

Lyme disease (LD) is a complex multisystem infection caused by *Borrelia burgdorferi* and is the most common vector-borne disease in the United States (2). The diagnosis of LD is based primarily on clinical findings, and treating patients with early disease solely on the basis of objective signs and a known exposure is appropriate. Laboratory tests may be a substantial aid to diagnosis when applied appropriately. Serologic testing is the most commonly used corroborative laboratory test and can be helpful in patients with clinical findings that are suggestive of later-stage disseminated LD. In all regards, it would be immensely useful for the management of LD to have available a test that would reflect infection. Unfortunately, current *B. burgdorferi* serologic tests based on detection of antibody to whole-cell antigens either do not change significantly following treatment or, when they do, the changes do not correlate with the presence or absence of a cure (8). It is difficult to detect *B. burgdorferi* by culture or PCR beyond very early disease, arthritis, or acrodermatitis chronicans atrophicans, and these methods often yield negative results in the face of disease manifestations that are clearly associated with persistent *B. burgdorferi* infection (12, 17, 18). Therefore, negative results in these tests may not exclude the possibility of persistent infection.

Posttreatment Lyme disease syndrome (PTLDS) designates

the condition of patients who suffer from chronic symptoms after adequate antibiotic therapy, even though evidence of a persistent infection is lacking. Common complaints include fatigue, headaches, myalgias, arthralgias, and cognitive impairment. The mechanism underlying this syndrome is unknown, and management of these patients is controversial, but antibiotic therapy appears not to be beneficial (11).

The *B. burgdorferi*-specific immune complex (IC) test has been used in early Lyme infection and has been suggested as a possible marker of active infection (5, 6, 16). One method uses polyethylene glycol (PEG) precipitation to isolate the IC from the serum (PEG-IC). The objective of this study was to examine the presence of *B. burgdorferi*-specific antibodies within serum PEG-ICs in patients with LD, patients with PTLDS, and controls and evaluate whether this test could be useful as a marker of active infection.

MATERIALS AND METHODS

Patients and controls. Cases included patients referred to the Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, Md., with early LD ($n = 6$), neurological LD ($n = 2$), Lyme arthritis (LA) ($n = 7$), and PTLDS ($n = 19$). Early-LD patients had erythema migrans and history of exposure in an area where the disease is endemic (four patients from Maryland and two from Virginia). Patients with LA had monoarticular or oligoarticular arthritis, exposure in an area where the disease is endemic (six patients from Maryland and one from New Jersey), and positive immunoglobulin G (IgG) antibody responses to *B. burgdorferi* by enzyme-linked immunosorbent assay (ELISA) and Western blotting (WB), interpreted according to the Center for Disease Control and Prevention (CDC) criteria (1). One neurological-LD patient had lymphocytic meningitis and facial palsy, while the other had peripheral neuropathy. Both had exposure in an area where LD is endemic (Maryland) and positive IgG antibody responses to *B. burgdorferi* by ELISA and WB. PTLDS patients had past histories of LD according to the CDC clinical definition (3),

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had past positive serologic analyses confirmed by IgG WB, and had persistent or intermittent symptoms for at least 6 months after appropriate antibiotic therapy for LD. Usual symptoms included widespread musculoskeletal pain and fatigue; memory and/or concentration impairment; and radicular pain, paresthesias, or dysesthesias. The onset of symptoms was coincident or within 6 months of initial *B. burgdorferi* infection, and the symptoms were significant enough to interfere with daily life activities. Other causes were excluded. Detection of *B. burgdorferi* DNA by PCR using the outer surface protein A (OspA) gene target and/or 16S ribosomal gene target from the genus *Borrelia* was negative in plasma and cerebrospinal fluid of PTLDS patients. None of the patients received the OspA vaccine (Lymerix). Their geographical distribution was as follows: four patients from Maryland, four from Massachusetts, three from New Jersey, two from Pennsylvania, and one each from Virginia, New York, Wisconsin, Delaware, California, and Connecticut.

Controls included healthy volunteers from the area of endemicity ($n = 18$) and OspA vaccinees ($n = 31$). Both groups had no previous history compatible with LD. Healthy volunteers had a negative Western blot to *B. burgdorferi* in the serum by the CDC criteria. OspA vaccinees had received at least two doses of the OspA vaccine (Lymerix) before the evaluation. The OspA vaccinee group included 11 females and 20 males, with a mean age of 49 years (range, 20 to 69 years). The mean time from the last dose of the vaccine to the study sample was 6 months.

All patients and controls had negative rapid plasma reagin in serum. The study was approved by the Institutional Review Board of the National Institute of Allergy and Infectious Diseases, and all patients and controls gave written informed consent.

Monkeys. Serum samples were obtained from rhesus macaques that were chronically infected with *B. burgdorferi* ($n = 3$), uninfected ($n = 10$), or vaccinated with OspA ($n = 7$). Chronically infected animals were inoculated with the JD1 strain of *B. burgdorferi* as described previously (9). Blood samples were obtained at several weeks postinfection and pooled for each animal, with week 22 being the earliest collection time and week 36 the latest. OspA-vaccinated animals were given inoculations with OspA/Al(OH)₃ with or without the adjuvant monophosphoryl lipid A, as described previously (13). Blood samples were obtained at 10 weeks ($n = 4$) and 6 weeks ($n = 5$) after the first vaccine dose.

PEG-IC isolation and dissociation. ICs were isolated by PEG precipitation and dissociated as previously described (4, 16). Briefly, 0.3 ml of serum was added to an equal amount of 7% PEG in 0.1 M sodium borate buffer (pH 8.4) and incubated for 2 h or overnight at 4°C. After 8,400-relative-centrifugal-force centrifugation for 10 min, the pellet was washed twice with 3.5% PEG in 0.1 M borate buffer, pH 8.4, and resuspended in 0.15 ml of 0.1 M borate buffer, pH 10.2. The elevated pH serves to dissociate the PEG-IC. Further antigen concentration was attempted for several PEG-IC preparations by neutralizing the dissociated PEG-IC with 3 M sodium acetate (pH 5.27), thereby reassociating the antigen-antibody complexes. These newly formed antigen-antibody complexes are capable of binding immunoprecipitating proteins conjugated to agarose beads as previously described (4). Briefly, to bind IgG-containing complexes, 0.1 ml of GammaBind G Sepharose (Pharmacia, Piscataway, NJ) was added to the above-mentioned neutralized PEG-IC sample, and the sample was placed on a reciprocal shaker for 1 h at 4°C. Binding of IgM complexes was attempted with the addition of 0.1 ml of mannan-binding protein (UltraLink; Pierce, Rockford, Ill.) and a similar 1-hour incubation. Protein L-agarose (Santa Cruz, CA) was then added to bind IgA and any other IgG or IgM antibodies that may not have bound to the other immunoprecipitating proteins. This antibody binding mixture was shaken overnight at 4°C and centrifuged at 8,400 relative centrifugal force for 15 min (beaded IC).

ELISA for PEG-ICs and serum antibodies to *B. burgdorferi*. Dissociated PEG-ICs were diluted 1:10, and unprocessed serum samples (diluted 1:100) were tested separately by IgG and IgM ELISA (MarDx, Carlsbad, Calif.), following the manufacturer's directions. ELISAs were considered to be positive when the optical density readings were greater than 3 standard deviations above the mean of at least 10 negative controls run on each plate.

Western blots. Identification of OspA from *B. burgdorferi* in the dissociated PEG-IC material was done by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions in either 12% or 4 to 12% precast Bis-Tris Gels with MOPS (morpholinepropanesulfonic acid) (Invitrogen Life Technologies, Carlsbad, CA). The beaded PEG-IC material was resuspended in Western blot reducing buffer, boiled to dissociate the antigens, and electrophoresed using the same precast gels. Proteins were transferred onto polyvinylidene difluoride membranes using the Pharmacia semidry Multiphor II NovaBlot (Amersham Pharmacia Biotech Inc., Piscataway, NJ) transfer apparatus. *B. burgdorferi* B31 sonicate (BioDesign International, Saco, ME) or recombinant OspA (provided by

TABLE 1. Samples evaluated using serum and PEG-IC ELISA for the presence of anti-*B. burgdorferi* IgG and IgM

Category	IgG ^a	IgM ^a
PTLDS	19 (39)	13 (31)
Early LD	6 (6)	5 (7)
LA	7 (12)	4 (8)
Neurologic LD	2 (6)	2 (6)
OspA vaccinees	31 (31)	13 (13)
Healthy volunteers	18 (18)	11 (12)
Total	83 (112)	48 (77)

^a The first number is the number of individuals tested in each group. Some participants had more than one sample analyzed, and the number of distinct samples tested is given within parentheses.

GlaxoSmithKline S.A., Gerval, Belgium, and John Dunn, Brookhaven National Laboratory, Upton, NY) were used as controls.

Membranes were probed using anti-OspA monoclonal antibodies H5332 (provided by Alan Barbour, University of California—Irvine, Irvine, CA) and C65550M (BioDesign International, Saco, ME), followed by peroxidase-conjugated goat anti-mouse IgG Fab-specific antibody A2304 (Sigma-Aldrich Corp., St. Louis, MO). A third anti-OspA monoclonal antibody was also used. This was MAB302 (Maine Biotechnology Services Inc., Portland, ME), biotinylated with E-Z link biotin hydrazide (Pierce Biotechnology, Inc., Rockford, IL) and detected with the Vectastain Elite ABC kit (Vector Laboratories, Inc., Burlingame, CA). This approach was introduced to avoid the need to use a secondary antibody to detect OspA. Membranes also were probed with peroxidase-conjugated rabbit polyclonal antibody against *B. burgdorferi* B65304P (BioDesign International, Saco, ME).

RESULTS

We analyzed 112 different samples (from 83 individuals) for the presence of anti-*B. burgdorferi* IgG and 77 distinct samples (from 54 individuals) for the presence of anti-*B. burgdorferi* IgM (Table 1). All samples were assessed in a blinded fashion as follows: the tubes were labeled with random numbers by one of the investigators, who was not involved in performing the assay; the assay was then performed by another investigator, who was blinded to the origin of the serum.

The results for both IgG and IgM ELISAs from unprocessed serum and PEG-IC tests are shown in Table 2. There was a high likelihood that samples that were positive by ELISA performed on unprocessed serum were positive by IC-PEG. When the results of all samples tested for IgG were evaluated, 94.7% (54/57) that were positive by serum IgG ELISA had a positive PEG-IC IgG ELISA result, while only 25% (11/44) of the samples that were negative by serum IgG ELISA had a positive PEG-IC IgG ELISA. In the PTLDS group, all samples with positive serum IgG ELISAs were also positive by PEG-IC IgG ELISA (26/26), while 64% (7/11) of the sera negative by IgG ELISA had a positive PEG-IC IgG ELISA result. In the OspA vaccinee group, 87.5% (14/16) of those unprocessed serum samples that were ELISA positive were also positive by PEG-IC. In the evaluation of IgM, the PTLDS group had 62.5% (10/16) of the serum IgM ELISA-positive samples also positive by PEG-IC IgM ELISA, while 23% (3/13) of the negative serum IgM ELISAs were positive by PEG-IC IgM ELISA. In the early-LD group, all negative serum IgM ELISA samples were also negative by PEG-IC IgM ELISA, while 75% (3/4) of the positive serum IgM ELISA samples were PEG-IC IgM ELISA positive. In the OspA vaccinee group, 27% (3/11) of the samples negative by IgM ELISA were positive by PEG-IC

TABLE 2. ELISA and PEG-IC IgG and IgM results

Category	Serum ELISA result	All samples ^a		First sample only ^b	
		PEG-IC IgG ELISA (no. positive/serum IgG ELISA)	PEG-IC IgM ELISA (no. positive/serum IgM ELISA)	PEG-IC IgG ELISA (no. positive/serum IgG ELISA)	PEG-IC IgM ELISA (no. positive/serum IgM ELISA)
All samples	Negative	11/44	7/42	6/35	5/28
	Positive	54/57	21/33	35/38	12/19
	Indeterminate ^c	6/11	1/2	5/10	0/1
PTLDS	Negative	7/11	3/13	3/5	2/5
	Positive	26/26	10/16	14/14	4/7
	Indeterminate ^c	2/2	1/2		0/1
Early LD	Negative	1/3	0/3		0/2
	Positive	1/2	3/4		2/3
	Indeterminate ^c	0/1	0/0		0/0
All Lyme ^d	Negative	10/18	4/21	5/9	2/8
	Positive	40/41	19/29	21/22	10/15
	Indeterminate ^c	3/4	1/2	2/3	0/1
OspA vaccinees	Negative	1/9	3/11		3/11
	Positive	14/16	2/2		2/2
	Indeterminate ^c	3/6	0/0		0/0
Healthy volunteers	Negative	0/17	0/10		0/9
	Positive	0/0	0/2		0/2
	Indeterminate ^c	0/1	0/0		0/0

^a Includes all results.

^b Uses only the first complete sample pair per patient or control. Where there are no data in these columns, they are the same as those indicated in the "All samples" column.

^c Refers to samples with equal numbers of positive and negative results (in cases where the sample was tested more than once).

^d Refers to all patients with Lyme disease and includes posttreatment Lyme disease syndrome, early Lyme disease, Lyme arthritis, and neurologic Lyme disease patients.

IgM ELISA, and both samples that were positive by serum IgM ELISA were also positive by PEG-IC. The proportions were similar if the results of only one sample per patient were considered. The precision values (frequency of obtaining the same result on repeat analysis of a specimen) (Table 3) for the IgG and IgM ELISAs in the serum were 65% and 75%, respectively, while the IgG and IgM PEG-IC precision values were 82% and 75%.

Next, we evaluated a subset of samples in an independent laboratory. The samples from 4 healthy volunteers, 5 patients with LA, 1 patient with late neurological LD, and 10 OspA vaccinees were run blinded, and the laboratory used the same process for IC dissociation. Both unprocessed serum and PEG-IC were assessed for the presence of anti-*B. burgdorferi* IgG by ELISA using the MarDx kits. The results were similar to the initial ones. Overall, 92% of the samples positive by serum IgG ELISA had a positive PEG-IC IgG ELISA result, while none of the samples that were negative by serum IgG ELISA had a positive PEG-IC IgG ELISA. For the OspA vaccinees, 87% of the samples positive by serum IgG ELISA had a positive PEG-IC IgG ELISA result.

TABLE 3. Precision values for IgG and IgM serum and PEG-IC ELISA

Assay	No. of samples	Precision (%) ^a
IgG serum	29	65
IgG PEG-IC	34	82
IgM serum	12	75
IgM PEG-IC	16	75

^a Precision is the frequency of obtaining the same result on repeat analysis of a specimen.

Results that were obtained with the monkey serum samples paralleled the results obtained in humans. All of the OspA-vaccinated monkey samples ($n = 9$) were positive by both serum IgG ELISA and PEG-IC IgG ELISA. These samples were negative for both serum IgM ELISA and PEG-IC IgM ELISA. Results obtained with serum specimens from infected monkeys ($n = 3$) were the same with both tests, while samples from control monkeys ($n = 10$) were negative in all tests.

We next addressed the question of whether serum IgG ELISA and PEG-IC IgG ELISA were independent tests. Because all but one healthy volunteer had negative serum ELISA results, we used the results from OspA vaccinees to assess the independence of the tests, as it is expected that vaccinees would have mostly (or only) free anti-OspA IgG antibody. Sixteen (52%) tested positive for serum IgG ELISA. Fourteen of the 16 samples positive by serum IgG ELISA were positive by PEG-IC IgG ELISA. Using Fisher's exact two-tailed test, we found that the frequency of positive PEG-IC IgG ELISA was significantly higher among ELISA-reactive than among ELISA-negative serum samples ($P = 0.0003$). The difference is also significant if the six serum samples with indeterminate ELISA results (samples that were tested twice and had different results in each test) are added to the positive samples ($P = 0.001$).

To address the question of concentration of immunoglobulins by the PEG-IC procedure, we measured the immunoglobulin contents of 19 serum and PEG-IC pairs by nephelometry (Beckman Array 360; Beckman Coulter, Inc., Fullerton, CA). Samples were assessed in a blinded fashion at the Clinical Pathology Laboratory of the Warren Grant Magnuson Clinical Center. The samples were prepared and diluted as described in Materials and Methods for use in the ELISA (PEG-ICs were

diluted 1:10, and sera 1:100). For PEG-ICs and sera, the mean IgG values were 111.69 mg/dl and 10.3 mg/dl, while the mean IgM values were 103.82 mg/dl and 1.63 mg/dl. Taking into account the initial steps used in the preparation of the PEG-IC (where samples were actually concentrated 2:1), we calculated the total amounts of IgG and IgM by multiplying the measured values by 100 for the serum samples and by 5 for the PEG-ICs. The serum IgG and IgM mean values were 1,030 mg/dl and 163 mg/dl, while the PEG-IC IgG and IgM mean values were 558.5 mg/dl and 519 mg/dl. Therefore, the IgM values in the PEG-ICs were increased by 3.2-fold while the IgG values were 50% less than the respective serum values.

Next, we tried to detect *B. burgdorferi* antigen in PEG-ICs from patients with active infection by immunoprecipitation followed by immunoblot analysis for the presence of OspA, using the anti-OspA monoclonal antibodies H5332 and C65550, as well as the polyclonal anti-*B. burgdorferi* antibody. We observed similar banding patterns in all patient and control groups in all of the probed membranes, with between two and three bands (depending upon the gel resolution) migrating around the 31-kDa OspA migration zone. These results were present in multiple experiments, and the use of the Gamma-Bind G Sepharose, mannan-binding protein, and protein L-agarose antibody binding beads in the antigen preparation (see Materials and Methods) failed to remove the nonspecific binding. Lowering the concentration of the primary H5332 anti-OspA monoclonal antibody from 1:10 to 1:100 did not allow any specific OspA bands to appear. The use of the biotinylated MAB302 anti-OspA antibody gave similar results. We also dissociated the PEG-ICs by using an acidic pH of 3.5 instead of the sodium acetate basic pH 10.2 buffer and tried the less reactive Western Lightning Chemiluminescence Reagent (Perkin-Elmer Life Sciences, Boston, MA) to prevent any nonspecific bands from possibly overwhelming any OspA-specific visualization, with no success. In a final attempt to obtain an OspA-specific band, proteins were transferred from the gels to the polyvinylidene difluoride membrane using a wet-transfer mode (Trans-Blot; Bio-Rad, Hercules, CA), followed by colorimetric development with TMB-1 component membrane peroxidase substrate (BioFX Laboratories, Owings Mills, MD). Using the last method, no bands, specific or nonspecific, appeared around the 31-kDa area in the PEG-IC lanes.

DISCUSSION

This study demonstrates that PEG-IC assays and serum ELISAs for antibodies to *B. burgdorferi* are not independent tests, as our results show that a positive PEG-IC ELISA result correlates strongly with a positive ELISA result in unprocessed serum, and that was true for a control population that was not expected to have circulating immune complexes. All of the previous studies of immune complexes in LD have compared patients to seronegative volunteers and did not use an uninfected seropositive control group, as represented by the OspA vaccinees included in our study. There is potential for long-term interference with diagnostic tests for Lyme disease in recipients of the vaccine, as antibodies against OspA can be present in a large percentage of individuals years after they receive the vaccine (10).

There are two possible explanations for our finding of the

positive PEG-IC in vaccinated individuals. The most likely possibility is that the method used for the preparation of the PEG-IC precipitates not only immune-complexed antibody but also a significant quantity of free antibody. This possibility, coupled with our finding that there is a much larger amount of immunoglobulin in the PEG-IC preparations than in the unprocessed serum, as used for the ELISA (111.69 mg/dl for IgG and 103.82 mg/dl for IgM in the PEG-ICs versus 10.3 mg/dl for IgG and 1.63 mg/dl for IgM in unprocessed serum), could explain the cases when positive PEG-IC tests occur in the face of a negative serum ELISA result. It can also explain the increased sensitivity of the PEG-IC test in early disease, instead of the exclusive freeing of complexed antibody to make the antibodies in the immune complexes accessible to measurement by ELISAs, as has been hypothesized (7, 14–16). One question regarding this hypothesis is why we would have results where the serum ELISA was positive but the PEG-IC was negative. That discrepancy occurred in 3 and 12 samples with the IgG and IgM ELISAs, respectively. When examined more closely, two of the IgG samples were tested more than once, and in both, the results were different at the second analysis (one had negative serum with a positive PEG-IC, while the other had both serum and PEG-IC positive). The other sample and all the samples showing inconsistent IgM results were tested only once. It is possible that the discrepancies are due to the intralaboratory variability of the test and would be resolved by retesting these samples.

Another potential explanation for the findings of positive PEG-ICs in the vaccinated group is the production of anti-idiotypic antibodies after vaccination, which would form circulating immune complexes with the anti-OspA antibodies. If this hypothesis were correct, the precipitated immunoglobulins would be part of immune complexes. As similar anti-idiotypic immune complexes could also be formed in patients after clearing of the infection, these complexes would still present a problem for the use of this test as a possible marker of infection, as precipitated antibodies would not necessarily represent antibody-antigen complex.

In conclusion, this study presents evidence contrary to the use of PEG-IC combined with standard ELISA as a marker of active infection in patients with persistent symptoms or in individuals who received the OspA vaccine.

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