

Original Article

Detection of 41-kDa bacterial flagellin protein by the lymphocyte transformation test-memory lymphocyte immunostimulation assay

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Abstract: Background/objectives: Diagnosis of human infection by various species of the bacterial genus *Borrelia* is mainly reliant on serological testing, polymerase chain reaction (PCR) or culture but such serological tests have been reported to have heterogeneous sensitivities, while *Borrelia* PCR and culture have been reported as being of modest diagnostic value. It has been suggested that the adjunctive use of the lymphocyte transformation test-memory lymphocyte immunostimulation assay (LTT-MELISA) may be helpful in this regard; however, the clinical usefulness of this assay has been questioned. The *Borrelia* immunodominant 41-kDa flagellin protein almost always gives rise to a marked human antibody response following infection. It was therefore decided to determine whether the LTT-MELISA detects the human antibody response to this antigen. Methods: Blood samples from consecutive patients with possible borreliosis attending a clinic were independently tested by both Western blots and LTT-MELISA. Results: After omitting cases with indeterminate Western blot results and equivocal LTT-MELISA results, multiple linear regression modelling demonstrated that the 41-kDa flagellin immunoglobulin (Ig) M level was predictable from two LTT-MELISA variables ($F_{2,51} = 5.981$, $P = 0.005$). Similarly, the corresponding 41-kDa IgG model also contained two LTT-MELISA variables ($F_{2,57} = 3.700$, $P = 0.031$). Conclusion: It is concluded that the LTT-MELISA appears to be able to detect the response to this antigen.

Keywords: Flagellin, lymphocyte transformation test, MELISA

Introduction

The bacterial genus *Borrelia* belongs to the phylum *Spirochaetota* [1]. These spirochaetes are Gram negative and use axial filamentous endoflagella to enable movement to take place in a corkscrew-like fashion [2, 3]. The endoflagellum of the bacterium is contained in the periplasmic space, amidst an outer envelope which provides protection, and comprises subunits of the globular protein flagellin; this protein has a molecular mass of 41 kDa [3]. The outer surface proteins (Osps) appear to play an important role in virulence, dissemination, tissue tropism and evasion of the host immune system by the bacteria [4, 5].

Many species, including *B. burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, *B. spielmanii* and *B. bavariensis*, are associated with the arthropod-borne zoonosis Lyme borreliosis [6]. Other spe-

cies, such as *B. recurrentis*, *B. duttonii* and *B. turicatae*, are associated with relapsing fever [7]. Clinical manifestations of Lyme borreliosis include dermatological lesions such as erythema migrans and acrodermatitis chronica atrophicans; arthritis; cardiac disorders such as conduction abnormalities, endocarditis, myocarditis and pericarditis; and neurological disorders such as polyradiculitis, meningoradiculoneuritis, (Garin-Bujadoux-Bannwarth syndrome), polyneuropathy, encephalitis, cerebral vasculitis and stroke [8-13]. Clinical manifestations of *Borrelia*-associated relapsing fever include, in the case of soft tick-borne relapsing fever, febrile episodes, chills, rigor, iritis, uveitis, acute respiratory distress, myocarditis, cranial nerve palsy and splenic rupture; and, in the case of louse-borne relapsing fever, febrile episodes, rigor, meningism, hepatomegaly, icterus, epistaxis, dermatological lesions, subconjunctival haemorrhage, acute respiratory distress,

splenic rupture, disseminated intravascular coagulation and coma [7, 14, 15].

Diagnosis of human infection by various *Borrelia* species is mainly reliant on serological testing, polymerase chain reaction (PCR) or culture [16]. However, the serological tests have been reported to have heterogeneous sensitivities, while *Borrelia* PCR and culture have been reported as being of modest diagnostic value [16, 17].

To address these difficulties, Valentine-Thon and colleagues developed a novel lymphocyte transformation test-memory lymphocyte immunostimulation assay (LTT-MELISA) using informative recombinant antigens [18]. Valentine-Thon and her colleagues reported that 86% of 90 LTT-MELISA positive patients were seropositive for borreliosis and symptomatic; the new assay was reported to have a specificity of almost 97% and a reproducibility of almost 93% [18]. A subsequent study by von Baehr and colleagues reported a sensitivity for clinically active borreliosis of this assay of over 89% and a specificity of almost 99% [19]. The latter group therefore suggested that this assay “could fill a gap in the difficult diagnostics of borreliosis” [20]. On the other hand, several groups have strongly argued against the use of this assay, partly on the basis that it has not been shown to be clinically useful [16, 21, 22], although this has been disputed [23, 24].

Given that the *Borrelia* immunodominant 41-kDa flagellin protein almost always gives rise to a marked antibody response following infection [25-29], it could be argued that if the LTT-MELISA were clinically useful, then at the very least it should detect this response. Accordingly, the aim of this study was to determine whether the LTT-MELISA detects the human antibody response to this antigen. This represents an extension of a previous LTT-MELISA study by our group, in which we reported that, out of 45 patients who were seronegative for borreliosis according to the serodiagnostic algorithm recommended by the Centers for Disease Control and Prevention (CDC), 19 (42%) were positive by the LTT-MELISA [30, 31].

Materials and methods

Subject samples

For clinical purposes, venous blood samples were obtained from 106 patients, 42 (40%) ma-

les and 64 females, of mean age 40.2 years (standard error 1.6 years), who presented consecutively to a clinic in the United Kingdom with a clinical history and symptomatology consistent the possibility of borreliosis. Inclusion criteria included being male or female; a history of one or more tick bites; a clinical history or physical signs of infection by *Borrelia*, without any clinical history or other evidence of an alternative aetiology, including any of the following: dermatological lesions such as erythema migrans; arthritis; cardiac disorders such as conduction abnormalities, endocarditis, myocarditis and pericarditis; and neurological disorders such as polyradiculitis, meningoradiculoneuritis, (Garin-Bujadoux-Bannwarth syndrome), polyneuropathy, encephalitis, cerebral vasculitis, stroke, relapsing unexplained pyrexia, meningism and cranial nerve involvement such as facial palsy. Patients for whom a non-*Borrelia*-related cause for their symptomatology was known were excluded. Part of each sample was centrifuged and the serum obtained sent for Western blot (immunoblot) analyses by IGeneX (Palo Alto, CA), while non-centrifuged whole blood was sent for LTT-MELISA assessments by LADR, Medizinisches Versorgungszentrum (Bremen, Germany). Both laboratories were blinded from each other in respect of these samples. This retrospective clinical audit received ethical approval from a Research Ethics Committee and was carried out according to the Declaration of Helsinki.

Outcome and predictor variables

The principal outcome variables were Western blot 41-kDa IgM and IgG levels. Each band intensity, x_i , was classified as follows: negative (-): $x_i = 0$ (that is, no band detected); indeterminate: $0 < x_i < c_i$; one-plus positive (+): $x_i = c_i$; two-plus or greater (++ to ++++): $x_i > c_i$; where c_i is the intensity of the calibration standard corresponding to x_i .

The predictor variables were the stimulation indices of wells in which peripheral blood mononuclear cells (PBMCs) were incubated with recombinant OspC from *B. afzelii*; recombinant OspC from *B. burgdorferi sensu stricto*; recombinant p41 from *B. garinii*; recombinant p41 from *B. afzelii*; recombinant p41 from *B. burgdorferi sensu stricto*; recombinant p100 from *B. afzelii*; recombinant peptide mix (OspA/VlsE from *B. afzelii*, *B. garinii* and *B. burgdorferi sensu stricto*); and full antigen lysate from *B. burgdorferi sensu stricto*.

The methodologies for carrying out the Western blots and the LTT-MELISA assessments are described, respectively, in the next two subsections.

Western blots

Ten microlitres of serum from each patient sample was aliquoted and tested using Western blot (immunoblot) strips prepared from a mixture of the B31 and 297 strains of *B. burgdorferi*, as previously described [32]. The B31 strain (vlsE^{B31}) was isolated from the adult *Ixodes dammini* tick vector by Burgdorfer and colleagues in 1982, while the 297 strain (vlsE²⁹⁷) was recovered from infected human cerebrospinal fluid by Steere and colleagues in 1983 [33-35]. Internal validation was carried out whereby each intensity of the 41-kDa IgM and IgG bands, x_i , was compared with the corresponding calibration standard intensity, c_i , and the result expressed on the ordinal scale described in the previous subsection.

LTT-MELISA

One million PBMCs in 1 mL medium from each of the subjects, and control samples, were incubated in a multi-well plate coated with recombinant *Borrelia* antigens at 310 K with 5% CO₂ for five days, as described previously [18, 36]. The recombinant antigens used are listed above and were present at three different dilutions. The samples were then exposed for five hours to methyl-³H-thymidine (Amersham Buchler, Brunswick) at a radioactive intensity of 3 μ C (specific activity 185 GBq/mmol), following which the uptake of the radiolabelled thymidine was determined using a 1450 MicroBeta TriLux microplate scintillation and luminescence counter (PerkinElmer, Shelton, CT). The stimulation index (SI) of a given well was calculated as the ratio of its radioactive count to the mean reading from three control sample wells. The cut-offs were as follows: SI < 2: negative; 2 \leq SI \leq 3: a possible reaction; SI > 3: positive.

Statistical analysis

Multiple linear regression analyses were carried out with the Western blot 41-kDa IgM and IgG levels in turn as the outcome or dependent variable, each treated as a scale variable and not including any cases in which the IgM or IgG results were reported as being indeterminate.

The LTT-MELISA variables given above were treated as predictor variables; any cases in which these variables were reported as being equivocal were not included. A backward method was used with a stepwise removal criterion of $P > 0.1$. The intercept was included in the analyses, corresponding in each case to a statistical error term. The statistical analyses were carried out using R v. 4.1.1 and JASP 0.16.2 [37, 38].

Results

41-kDa IgM

For the Western blot 41-kDa IgM data, the regression analysis yielded the following statistically significant model ($F_{2,51} = 5.981$, $P = 0.005$):

$$41\text{-kDa IgM} = 2.145 + 0.602x_1 - 0.099x_2$$

The variable x_1 corresponded to the LTT-MELISA recombinant p41 from *B. garinii* while x_2 corresponded to the LTT-MELISA recombinant peptide mix (OspA/VlsE from *B. afzelii*, *B. garinii* and *B. burgdorferi sensu stricto*). The intercept term was statistically significant, as were the coefficients of x_1 (standard error = 0.212; $P = 0.006$) and x_2 (standard error = 0.034; $P = 0.006$).

41-kDa IgG

The regression analysis also yielded a statistically significant model for the Western blot 41-kDa IgG data ($F_{2,57} = 3.700$, $P = 0.031$), as follows:

$$41\text{-kDa IgG} = 3.021 - 0.350x_3 + 0.251x_4$$

The variable x_3 corresponded to the LTT-MELISA recombinant OspC from *B. burgdorferi sensu stricto* and x_4 corresponded to the LTT-MELISA recombinant p100 from *B. afzelii*. Again, the intercept term was statistically significant, as was the coefficient of x_4 (standard error = 0.117; $P = 0.036$). The standard error of the coefficient of x_3 was 0.179 ($P = 0.055$).

Discussion

The first main result of this study is that the 41-kDa flagellin IgM level is predictable from LTT-MELISA variables. Thus, the LTT-MELISA appears to be able to detect the early immunological response to this antigen.

The second main result is that the 41-kDa flagellin IgG level can also be predicted from LTT-MELISA variables. It follows that the LTT-MELISA appears to be able to detect the late immunological response to this antigen.

Taken together, both of the above results are consistent with previous reports that the LTT-MELISA indexes active borreliosis and so has diagnostic value in cases which are clinically or serologically ambiguous [18-20, 30].

Turning to the predictor variables found in this study, it is noteworthy that one of the two predictor variables for the 41-kDa flagellin IgM was an OspA/VisE recombinant peptide mix, while one of the two predictor variables for the 41-kDa flagellin IgG was a recombinant OspC. This is of relevance in relation to the serological testing which tends to be used for the detection of borreliosis. More traditional immunoassay techniques, such as the enzyme-linked immunosorbent assay (ELISA) and enzyme immunoassay (EIA), require binding by the detecting or primary antibodies to the surface bacterial antigens and are favoured by the CDC in its serological testing guidelines for borreliosis [39]. However, immunofluorescence experiments have demonstrated that *B. burgdorferi* OspA, OspB and OspC have only limited exposure at the bacterial surface [40]. It is possible that this accounts for the heterogeneous sensitivity of such serological testing in borreliosis. In contrast, LTT-MELISA relies on the ability of the bacterial antigens to transform lymphocytes; the change in activity of activated lymphocytes is assayed [18]. Thus, it is plausible that the LTT-MELISA might offer an improved diagnostic capability.

One shortcoming of this study is that it did not involve investigation by microscopy techniques. Future research should include such techniques in conjugation with appropriate immunofluorescent markers.

Flagellar antigens can be helpful diagnostically owing to their high discriminative value; indeed, the 41-kDa antigen has been incorporated into an antigen biochip for potential diagnostic use in borreliosis for this reason [41].

In *Borrelia*, and indeed most spirochaetes, flagellin proteins create an inner flagellar core (FlaB proteins) and an outer layer (FlaA pro-

teins), with the 41-kDa flagellin being principally associated with the FlaB protein [42, 43]. However, the 41-kDa flagellin is not only associated with spirochaetal periplasmic flagella but can be found in many non-spirochaetal bacterial species. For example, purified flagella of *Bartonella clarridgeiae* have been reported to be built from a 41-kDa polypeptide [44]. Thus, LTT-MELISA may have a role to play in the diagnosis of non-borrelial bacterial infections. It may also be worthwhile investigating the possible role of LTT-MELISA as a prognostic biomarker; in a recent systematic review, anti-flagellin antibodies comprised one of just two serological biomarkers with statistically significant prognostic potential in relation to developing severe Crohn's disease [45].

Disclosure of conflict of interest

None.

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Detection of flagellin by LTT-MELISA

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