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ORIGINAL INVESTIGATION

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An improved recombinant IgG immunoblot for serodiagnosis of Lyme borreliosis

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Abstract We have previously described the use of the following recombinant antigens for serodiagnostic immunoblots: p83/100, p39, OspC and p41 (flagellin) internal fragment [Wilske et al. (1993) Med Microbiol Immunol 182:255–270; Rössler et al. (1997) J Clin Microbiol 35:2752–2758]. In our currently used immunoblot p83/100 is derived from strain PKo (*Borrelia afzelii*), p39 (BmpA) and OspC from strains PKa2 (*B. burgdorferi* sensu stricto), PKo and PBi (*B. garinii*), respectively; the p41 (flagellin) internal fragments were cloned from strains PKo and PBi. In this study we describe the use of two additional recombinantly expressed highly immunogenic proteins Osp17 (derived from PKo) and p58 (derived from PBi). A clinically well-defined panel of sera from 147 Lyme borreliosis patients and 139 controls previously tested by a standardized whole cell lysate immunoblot [Hauser et al. (1997) J Clin Microbiol 35:1433–1444] was investigated in the recombinant immunoblot without (old recombinant immunoblot) and with Osp17 and p58 (new recombinant immunoblot) for IgG antibodies. The sensitivity of the recombinant IgG immunoblot for diagnosis of stage II and stage III could be significantly improved by addition of Osp17 and p58 without loss of specificity. With the exception of sera from patients with erythema migrans the diagnostic sensitivity is comparable to the whole cell lysate IgG immunoblot. The main advantage of the recombinant immunoblot is the easy identification of diagnostic bands, whereas the identification of bands in the whole cell lysate immunoblot is difficult. The recombinant immunoblot is especially suitable where large series of sera need to be investigated.

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Introduction

Lyme borreliosis (LB) is a multisystem disorder involving predominantly skin, nervous system and joints [14]. It is caused by *Borrelia burgdorferi* sensu lato (s.l.) [3] and is a global tick-borne disease specifically transmitted by *Ixodes pacificus* and *I. scapularis* in North America and *I. ricinus* and *I. persulcatus* in Eurasia. *B. burgdorferi* s.l. consists of three different human pathogenic species, *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii* [2, 4]. The heterogeneity of the humanpathogenic Lyme disease spirochetes in Europe [2, 6, 7, 15, 16, 19] is the cause of considerably heterogeneous antibody patterns in Western blots depending on the strains used as antigen [1, 5, 8, 17, 23].

We have previously developed a standardized whole cell lysate Western blot using three different strains (PKa2, PKo and PBi, belonging to the three species *B. burgdorferi* s.s., *B. afzelii* and *B. garinii*, respectively) as antigens. The Western blots have been standardized using 17-cm-long gels, a monoclonal antibody panel for identification of specific *Borrelia* proteins and a reference serum recognizing a broad panel of *Borrelia* proteins throughout the study [8]. This study has shown that reliable interpretation criteria must be developed for individual strains and Western blot conditions. In addition, differences in the sensitivity have been observed for certain homologous proteins of different strains [for example for BmpA (p39) and OspC] and the *B. afzelii* strain exclusively expressed Osp17, a highly sensitive antigen for antibody detection in late manifestations [8, 11, 13].

The conventional blot is difficult to evaluate; one needs very experienced personnel for reliable identification of bands. It is much easier to evaluate blots using recombinant proteins. This is especially important if

large numbers of sera need to be investigated. In addition, besides easier identification of antigenic bands, the recombinant immunoblot has the advantage that homologous proteins of different strains can be used on one blot.

We have previously described the use of the following recombinant antigens for serodiagnostic immunoblots: p83/100, p39, OspC and p41 (flagellin) internal fragment (p41i) [13, 18, 20].

In this study we describe the use of two additional recombinantly expressed highly immunogenic proteins, Osp17 and p58. We investigated whether the sensitivity of the currently used recombinant immunoblot (containing p83/100, p39, OspC and p41i) could be improved when Osp17 and p58 were included as antigens.

Material and methods

Borrelia sp. strains and cultivation

B. burgdorferi s.l. strains were cultivated at 33 °C in modified Kelly medium (MKP) [12] as described. Strains PKo (*B. afzelii*) and PBi (*B. garinii*) used in this study have been described previously [19].

Cloning of the *p58* gene, recombinant expression and purification

Tryptic peptides obtained from the 58-kDa protein of PKo were N-terminally sequenced. Using oligonucleotides corresponding to these sequences we were able to amplify a fragment of the *p58* gene by PCR. Following essentially the same strategy as for Osp17 [11], the PCR fragment of the *p58* gene was labeled with digoxigenin-11-dUTP (Boehringer Mannheim, Mannheim, Germany) and used as a probe. The *p58* gene was detected on a *Hin*dIII-fragment of *B. afzelii* PKo DNA. This fragment was cloned in pUC 18 and the sequence of *p58* of PKo was determined. Primers according to the 5′ and 3′ ends, respectively, were used to amplify the *p58* genes from strain PBi.

The nucleotide sequences and deduced amino acid sequences of *p58* of strain PKo and PBi were compared using the gap program of the GCG program package. The *p58* sequence data will appear in the EMBL/GenBank/DDBJ Nucleotide Sequence Data Libraries under the accession numbers AJ250012 and AJ250013 for strains PKo and PBi respectively.

The *p58* genes were cloned in pUHE 21 with five His residues attached at the C terminus and without the leader peptide. The expression of *p58* in recombinant *Escherichia coli* (XL1Blue) was induced by addition of isopropyl-β-D-thiogalactopyranoside.

Pelleted cells were resuspended in buffer A (50 mM sodium phosphate buffer pH 8.0, 500 mM NaCl, 1 mM imidazole) plus 20 µl benzonase (Merck, Darmstadt, Germany) and ruptured using a French Press (SLM AMINCO). p58 was purified using an FPLC-System (Pharmacia Biotech, Freiburg, Germany). In the first purification step, the supernatant was filtered and subjected to affinity chromatography on a $NiSO_4$-loaded IMAC column (Fractogel EMD Chelat, Merck, Darmstadt, Germany) using buffer A. Elution was done with a linear gradient of 1–500 mM imidazole in buffer A. The p58-containing fractions were pooled and dialyzed against buffer B (25 mM TRIS-HCl pH 8.5). In the second step purification was performed by anion exchange chromatography (Mono Q, Pharmacia Biotech). Elution was done with a linear gradient of 0–250 mM NaCl in buffer B. The p58-containing fractions were pooled and dialyzed against buffer C (25 mM TRIS-HCl pH 8.0, 150 mM NaCl) and purified by size exclusion chromatography (Superdex 75 26/60, Pharmacia Biotech) in buffer C. Purification was controlled by SDS-PAGE (see below) and immunoblotting with immune serum against *E. coli* as described [11, 13].

Sera from patients and control sera

In this study we used a clinically well-defined panel of sera from patients previously used in the study of Hauser et al. [8]. Sera from 66 patients with erythema migrans (unselected from a therapeutical trial), sera from 42 neuroborreliosis patients (stage II) with positive intrathecal *Borrelia*-specific antibody production and 39 patients with late manifestations (29 with acrodermatitis and 10 with Lyme arthritis). Negative controls comprised 118 sera from blood donors and 11 sera from syphilis patients (stage II and III) and 10 sera positive for rheumatoid factor (RF).

Rabbit immune serum against p58

Rabbits were immunized three times subcutaneously with purified recombinant p58 as previously described for production of polyclonal antibodies against recombinant OspC [21].

SDS-PAGE and immunoblotting

SDS-PAGE and immunoblotting using whole cell lysates were performed as previously described [8, 19]. Whole cell SDS lysates of washed bacteria were separated by SDS-PAGE (15% polyacrylamide gel) and proteins were blotted to nitrocellulose. The recombinant immunoblot was performed as described for recombinant BmpA using Immobilon-P polyvinylidene difluoride membrane (Millipore, Eschborn, Germany) [13].

The protein transfer was controlled by staining the membranes with Ponceau-S solution (Serva, Heidelberg, Germany). After destaining and blocking blots were reacted with the different antibodies. Human sera were diluted 1:200, bound IgG antibodies were detected with horseradish peroxidase (HRPO)-labeled anti-human IgG purchased from Dakopatts (Copenhagen, Denmark). The rabbit immune serum was tested in dilutions 1:1000, 1:10,000 and 1:100,000, goat immune serum against rabbit immunoglobulins for detection of immunocomplexes was obtained from Dakopatts.

Whole cell lysate immunoblot

A whole cell lysate immunoblot using *B. afzelii* strain PKo as antigen was performed as reference immunoblot for sera from patients and controls [8, 10].

Recombinant immunoblot

Two different immunoblot strips were used. One consisted of p83/100 derived from strain PKo (*B. afzelii*), p39 (BmpA) and OspC from strains PKa2 (*B. burgdorferi* s.s.), PKo and PBi (*B. garinii*) respectively and p41i from strains PKo and PBi [13, 18, 20]. The second strip contained Osp17 (derived from strain PKo) [11] and p58 (derived from strain PBi). These antigens were described as highly sensitive antigens for IgG antibody detection. According to previous evaluation of whole cell lysate immunoblots frequencies of IgM antibodies against Osp17 and p58 were not significantly higher in patients compared to negative controls [8]. Therefore, we developed only an IgG immunoblot.

Statistics

For statistical analysis Fisher's exact test for dichotomous variables was performed.

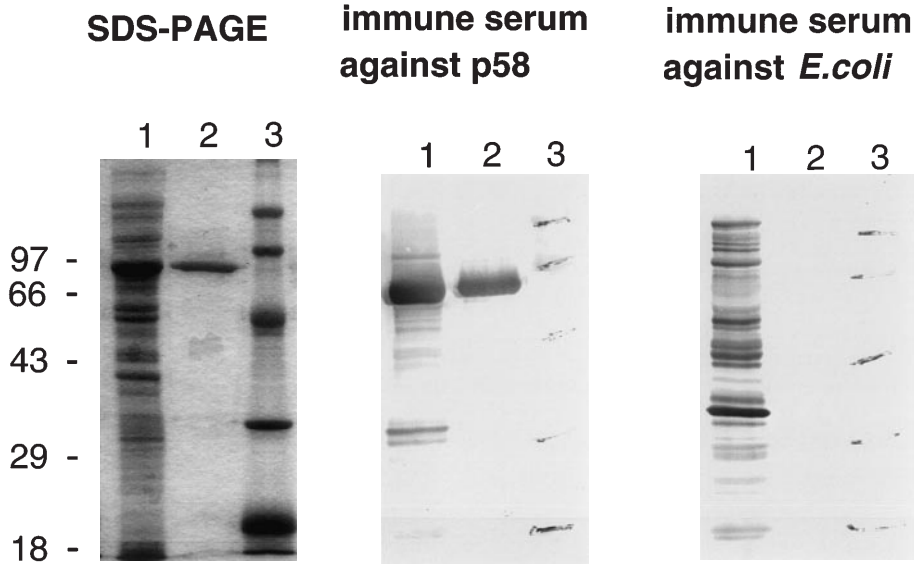

Fig. 1 Purification of recombinant p58; *lane 1* recombinant *E. coli* (whole cell lysate); *lane 2* purified p58; *lane 3* marker proteins, molecular weights in kDa

Results

Expression and purification of recombinant p58

A clone which effectively expressed p58 was obtained from strain PBi. Purified p58 was obtained by chromatography without contaminating other proteins. Even in the immunoblot with a high titered immune serum against *E. coli* no contaminating proteins were detectable and the purified protein was immunologically reactive (Fig. 1).

Improvement of antibody detection by addition of Osp17 and p58 as recombinant antigens to the IgG immunoblot

Comparison of different IgG immunoblots is shown in Table 1. The immunoblot was regarded positive when at least two bands were reactive. The three immunoblots were: (i) old recombinant immunoblot (p83/100, p39, p41i, OspC, partially derived from different strains as described above), (ii) new recombinant immunoblot (Osp17 and p58 in addition to the proteins of the old one), and (iii) the whole cell lysate blot using strain PKo, diagnostic bands, p83/100, p58, p43, p39, p30, OspC, p21, p17, and p14 [8, 10]. Examples for the old recombinant immunoblot and the p58/Osp17 immunoblot are given in Fig. 2a, b, respectively.

For all blots a one-band criterion (at least one of the indicated bands positive) was not acceptable since the specificity was only about 90% (data not shown) in contrast to a specificity of 97.5% when the two-band criterion was used. In addition, according to general German industrial norm (DIN) recommendations for the immunoblot (DIN 58967, part 4) at least two bands should be reactive if the immunoblot is regarded positive. We found a considerable increase in sensitivity when the new recombinant blot was used instead of the old recombinant one. The new recombinant blot was comparable in sensitivity (difference not significant) to the blot using whole cell lysate antigens with the exception of sera from patients with erythema migrans (difference highly significant, $P < 0.005$).

Table 2 summarizes the reactivities of the different recombinant proteins in comparison with the respective natural proteins. No significant differences were observed between the recombinant and the respective

Table 1 Comparison of different IgG-immunoblots

Group	No. of sera	Number of positive sera		
		New recombinant blot	Old recombinant blot	Whole lysate blot
Erythema migrans	66	7 (10.6%)	3 (4.5%)	22 (33.3%)
Neuroborreliosis[a]	42	19 (45.2%)	12 (28.6%)	24 (57.1%)
Late LB[b]	39	38 (97.4%)	29 (74.4%)	39 (100.0%)
Controls[c]	139	3 (2.2%)	3 (2.2%)	3 (2.2%)

[a] Neuroborreliosis (stage II)
[b] Acrodermatitis or arthritis (stage III)
[c] Blood donors ($n = 118$), patients with syphilis ($n = 11$), sera with positive rheumatoid factor ($n = 11$)

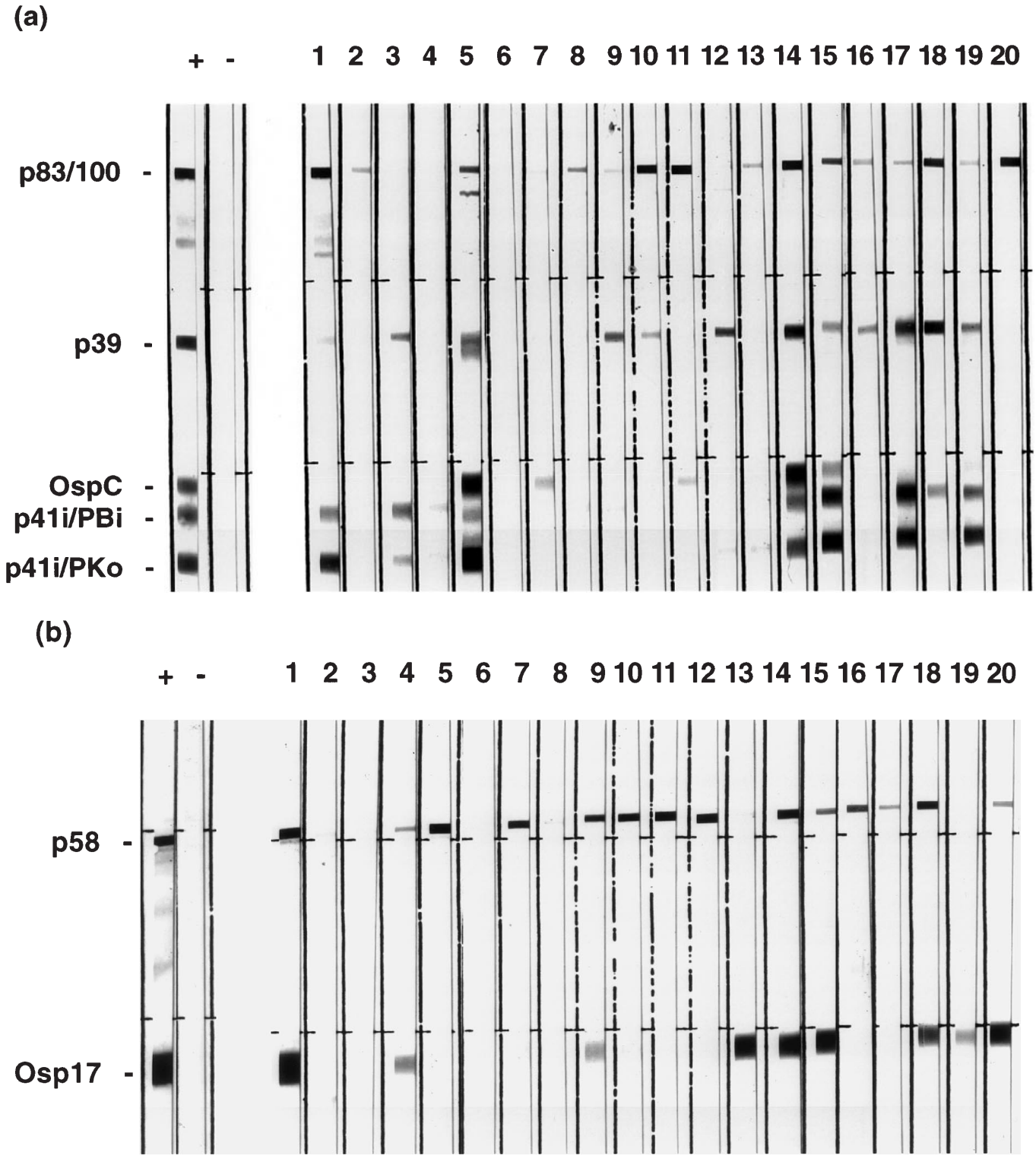

Fig. 2 IgG immunoblot with sera from patients with neuroborreliosis stage II using the following recombinant antigens: **a** p83/100, p39, OspC and p41i, and **b** p58 and Osp17 (+ positive controls, – negative controls, *1–20* sera from patients)

natural proteins in stage II and stage III patients. However, for the diagnosis of erythema migrans (stage I) recombinant p58 was significantly less sensitive ($P < 0.005$) compared to the respective natural protein.

Table 2 Reactivity of selected recombinant and natural *Borrelia* proteins in the IgG immunoblot (percentage of positive sera) (*p41i* internal flagellin fragment, *p41* full length flagellin, *nat* natural, *rec* recombinant)

Group	p100		p58		p41i	p41	p41i	p39		OspC		Osp17	
	nat PKo	rec PKo	nat PKo	rec PBi	rec PKo	nat PKo	rec PBi	nat PKo	rec B31/PKo/PBi	nat PKo	rec B31/PKo/PBi	nat PKo	rec PKo
EM $n = 66$	8	11	26	2	5	33	0	6	0	6	9	27	15
NB II $n = 42$	21	33	40	40	17	45	14	36	36	19	14	38	36
Late LB $n = 39$	79	67	97	92	69	74	41	85	51	21	10	92	85
Negative controls $n = 139$	0	6	3	1	3	22	2	1	0	1	0	4	1

Discussion

The theoretical consideration that Osp17 and p58 should be important recombinant antigens for serodiagnosis [9] could be verified experimentally by Western blot analysis of sera from Lyme disease patients. We have shown that addition of p58 and Osp17 considerably increased the sensitivity of the recombinant immunoblot.

Recombinant and natural Osp17 as well as p58 showed about the same sensitivity in patients with stage II and III manifestations (neuroborreliosis, acrodermatitis and Lyme arthritis). It is unclear why in erythema migrans patients the recombinant p58 is considerably lower in sensitivity than the respective natural antigen. An explanation might be that in the early stage antibodies are primarily directed against conformational epitopes not present in the recombinant protein or that strain heterogeneity may play a role. The recombinant p58 was derived from a *B. garinii* strain, while erythema migrans is mainly caused by *B. afzelii* strains. Inclusion of different homologous recombinant p58 and perhaps also Osp17 derived from different strains might further increase sensitivity of the recombinant blot as shown previously for other *Borrelia* proteins like OspC and p39 [13, 20]. The main disadvantage of the new recombinant IgG blot versus the whole cell lysate IgG blot is its lower sensitivity in stage I. In contrast IgM immunoblots are comparable in sensitivity for both whole cell lysate and recombinant antigens (about 40% of clinically diagnosed cases with erythema migrans were seropositive with either of the tests) [8, 20]. It should also be kept in mind that erythema migrans is primarily a clinical diagnosis, and even when highly sensitive tests are used patients are often seronegative. The main advantage of the recombinant blot is better standardization and easy identification of diagnostic bands. This is especially important for routine laboratory examinations and investigation of large serum panels. In addition, proper identification of antibody reactivities is important for discrimination of antibodies induced by natural infections and *Borrelia* vaccines.

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