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(54) Titre : PROTEINES A ACTIVITE IMMUNOLOGIQUE PROVENANT DE BORRELIA BURGENDORFERI, TROUSSES DE DOSAGE CONTENANT CES PROTEINES ET PERMETTANT LA DETECTION D'ANTICORPS DANS DES MILIEUX LIQUIDES, ET UTILISATION DE CES PROTEINES COMME VACCINS CONTRE LES INFECTIONS CAUSEES PAR DIFFERENTES SOUCHES DE BORRELIA
(54) Title: IMMUNOLOGICALLY ACTIVE PROTEINS FROM BORRELIA BURGENDORFERI, RELATED TEST KITS WHICH CONTAIN THESE PROTEINS AND ARE SUITABLE FOR DETECTING ANTIBODIES IN TEST FLUIDS, AND THE USE OF THESE PROTEINS AS VACCINES AGAINST INFECTIONS CAUSED BY BORRELIA STRAINS

(57) **Abrégé/Abstract:**

Various immunologically active proteins from *Borrelia burgdorferi* have been prepared by genetic manipulation in microorganisms. To do this, the specific DNA sequences were selected from a *B. burgdorferi* gene bank using suitable screening methods, or were prepared directly by DNA amplification using selected hybridization probes, and were placed under the control of inducible promoters such as, for example, the lac promoter. It has been possible, owing to description of efficient purification methods for the expressed antigens, to provide the proteins in a suitable way. These proteins can be used to produce specific and sensitive diagnostic assay kits. The specific combination of the immunologically active proteins makes precise diagnosis possible. Furthermore, monoclonal antibodies have been generated and are used as reagents for detecting pathogens directly in test samples or after cultivation. The *Borrelia burgdorferi*-specific DNA sequences can be employed for direct detection of the pathogen in patients' samples (for example by means of the PCR reaction).

Abstract of the disclosure

Various immunologically active proteins from *Borrelia burgdorferi* have been prepared by genetic manipulation in microorganisms. To do this, the specific DNA sequences were selected from a *B. burgdorferi* gene bank using suitable screening methods, or were prepared directly by DNA amplification using selected hybridization probes, and were placed under the control of inducible promoters such as, for example, the lac promoter. It has been possible, owing to description of efficient purification methods for the expressed antigens, to provide the proteins in a suitable way. These proteins can be used to produce specific and sensitive diagnostic assay kits. The specific combination of the immunologically active proteins makes precise diagnosis possible. Furthermore, monoclonal antibodies have been generated and are used as reagents for detecting pathogens directly in test samples or after cultivation. The *Borrelia burgdorferi*-specific DNA sequences can be employed for direct detection of the pathogen in patients' samples (for example by means of the PCR reaction).

Immunologically active proteins from *Borrelia burgdorferi*, assay kits which contain these proteins and are suitable for detecting antibodies in test fluids, and the use of these proteins as vaccines against infections caused by *Borrelia* strains.

10 Lyme borreliosis is the commonest infectious disease of humans transmitted by ticks in the Federal Republic of Germany. In contrast to Russian spring-summer encephalitis (RSSE) which is likewise transmitted by ticks, Lyme borreliosis is not confined to a few endemic areas but occurs in all the states of the FRG. Infestation of the main vector in Europe, *Ixodes ricinus*, with the pathogen of Lyme borreliosis, the spirochete *Borrelia burgdorferi*, in Southern Germany is about 20% of adults, about 10% of nymphs and about 1% of larvae. The main vector in the USA, *Ixodes dammini*, may be up to 100% infected by *Borrelia* in highly endemic areas.

20 *B. burgdorferi* belongs to the family of spirochetes. Spirochetes are spiral bacteria 8-30 μm long. They consist of an outer coat, the endoflagella in the periplasm and the protoplasmic cylinder. The protoplasmic cylinder is a complex of cytoplasm, internal cell membrane and peptidoglycan. Representatives of the spirochetes which are pathogenic for humans include, beside *B. burgdorferi*, the *Borrelia* of relapsing fever (for example *B. recurrentis*), the pathogen of syphilis (*Treponema* (T.) *pallidum*) and the *Leptospira*. As a result of the close immunological
30 relationship of the pathogens, cross-reactions are a problem in the serological detection of antibodies in cases of syphilis and Lyme borreliosis with assays currently available.

Infection with *B. burgdorferi* results in a complex clinical picture which can, similarly to syphilis, be divided into three different stages. The principal manifestations are:

- Early phase: Stage I Erythema migrans
- Bannwarth's lymphocytic
- meningoradiculitis (LMR)
- Borrelia lymphocytoma
- Late phase: Stage III Lyme arthritis
- Acrodermatitis chronica
- atrophicans (ACA)
- Chronic Borrelia
- encephalomyelitis

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Less common clinical manifestations are: carditis, myositis, iritis and panophthalmitis. Transmission by the pathogen crossing the placenta is possible but to date only a few cases of congenital Lyme borreliosis have been recorded. The various stages may occur singly or in combination. *B. burgdorferi* infection may also have a subclinical course. Epidemiological studies on 375 clinically confirmed cases show some peculiarities in the age and sex distribution of the various clinical manifestations. Thus, patients with Erythema migrans were commonest in the 30 to 60 year age group. Neurological manifestations showed two peaks with age: the first in children and young people up to 20 years of age, and the second in 40 to 70 year-olds. Lyme arthritis was observed to be commonest in 30 to 60 year-olds. Patients with ACA were never below 30 years of age. ACA affects women distinctly more often than men. Serological testing showed predominantly positive IgM findings in patients with Erythema migrans, and predominantly positive IgG findings when there were neurological manifestations, in an immunofluorescence assay. With the late manifestations of ACA and Lyme arthritis, the IgG

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titers were regularly elevated, and IgM antibodies were now detectable only in exceptional cases.

10 Available for diagnosis are both pathogen detection and antibody detection. Pathogen detection in material from patients (skin biopsies, CSF, puncture fluids) is recommended especially in the early stage (Erythema migrans) when antibody detection is frequently negative. However, a complex nutrient medium is required for culturing *B. burgdorferi* (Preac-Mursic, V.; Wilske, B.; Schierz, G. (1986): European *Borrelia burgdorferi* isolated from humans and ticks - culture conditions and antibiotic susceptibility. Zbl. Bakt. Hyg. A 163, 112-118) and cultivation is therefore restricted to special laboratories. In addition, a time of up to 5 weeks is required to isolate the pathogen. *B. burgdorferi* is isolated from skin samples in 50-70% of cases with cutaneous manifestations and in 3-5% of cases with neuroborreliosis (Preac-Mursic, V.; unpublished results).

20 Antibody detection (IgM, IgG) is carried out on serum and, when there are neurological manifestations, also from CSF. The serological finding depends on the stage of the disease, the duration of the symptoms and any antibiotic therapy which has already been applied. Thus, antibody detection with assays available to date is successful only in 20-50% of cases with Erythema migrans, in 50-90% of cases with neurological manifestations and in 90-100% in cases with ACA and arthritis.

30 Therapy of Lyme borreliosis is predominantly carried out with penicillin G, tetracyclines, erythromycin or cephalosporins. Although Lyme borreliosis frequently resolves spontaneously in the early stages, even then late manifestations are not ruled out. This is why therapy in the early stage is indispensable. In addition, clinical resolution after antibiotic therapy

can be achieved when there are late manifestations only in some of the cases (for example only about 50% of cases with Lyme arthritis).

10 This is why Lyme borreliosis should be diagnosed as early as possible. Since (as already explained) pathogen isolation is costly, time-consuming and, moreover, not always successful, better serodiagnostic assays ought to be developed. The methods used to date (immunofluorescence assay (IFA), indirect hemagglutination assay (IHA), enzyme-linked immunosorbent assay (ELISA)) frequently fail in the early stages. The anti-
20 gens employed for these assays are all *B. burgdorferi* cells or whole-cell ultrasonicates. The use of different *B. burgdorferi* strains as antigen in the ultrasonicate ELISA leads to differing test results. Immobilization of cells on slides or ultrasonicate antigen on microtiter plates is followed by incubation with serum or CSF and detection of the *Borrelia*-specific antibodies with a second fluorescence- or
20 peroxidase-labeled antibody of the appropriate immunoglobulin class. The reaction is then quantified either in a fluorescence microscope (IFA) or after a color reaction in a photometer (ELISA).

30 Broad cross-reactions of the pathogen *B. burgdorferi* with other bacterial pathogens, especially with *T. pallidum*, the syphilis pathogen, is a problem for the specificity of the assays. Since the assay antigens generally consist of lysates of the whole pathogen there is also detection of antibodies against so-called common antigens (Hansen, K.; Hindersson, P.; Pedersen, N.S. (1988): Measurement of antibodies to the *Borrelia burgdorferi* flagellum improves serodiagnosis in Lyme disease. *J. Clin. Microbiol.*, 26, 338-346). Common antigens are widely distributed proteins with highly conserved sequences, that is to say the common antigens of *Borrelia*, *Treponema* as well as many other bacteria

have common epitopes. Besides this, false-positive reactions may occur in the IgM-IFA or IgM-ELISA when the sera have rheumatoid factor activity. Therefore, in order to make the assays more specific, in the detection of IgG and IgM antibodies a preabsorption of the sera with a *Treponema* ultrasonicate, and additionally for the detection of IgM antibodies also absorption with rheumatoid factor absorbent, is carried out.

10 An object of the present invention is therefore to provide immunologically active proteins from *Borrelia burgdorferi* which are used in an assay kit which does not have the abovementioned disadvantages. An additional aim is that this assay kit makes it possible rapidly and reliably to detect antibodies directed against *Borrelia burgdorferi*.

20 Another object of the present invention is to provide monoclonal antibodies which are directed against particular immunologically active proteins from *Borrelia burgdorferi*. A further aim is to provide immunologically active proteins which are suitable as vaccines against infections caused by *Borrelia* strains.

30 Testing of patients' sera from different stages of the disease of Lyme borreliosis in a Western blot, and testing of non-Lyme borreliosis patients (especially syphilis patients) for cross-reactivity with *B. burgdorferi* resulted in the finding of immunologically active proteins (*B. burgdorferi* antigens) which, on the one hand, elicit a good antibody response after infection and, on the other hand, show a low cross-reactivity with sera which are not *B. burgdorferi*-positive (Example 1). It emerged that a particular strain of *B. burgdorferi* which has the internal laboratory identifier PKo and which was deposited at the Deutsche Sammlung für Mikroorganismen (DSM) under

No. 5662 possesses, inter alia, an immunodominant protein in the molecular-weight region about 22 kD (pC protein). The molecular weight of the proteins according to the invention was determined by methods known per se, in particular by SDS gel electrophoresis. It was found that this protein is immunodominant for the IgM response. This protein is not expressed in the same way in all *B. burgdorferi* strains. This immunologically active protein (pC protein) was prepared by genetic manipulation according to the invention (Example 3).

Other immunologically active proteins (antigens) which are particularly suitable for use in assay kits were also prepared in generally accessible and commercially available *Escherichia coli* cells such as, for example, strains JM 105 (Pharmacia) or DH 5 (Gibco-BRL). To do this, the *B. burgdorferi* DNA fragments coding for these proteins were isolated and subsequently inserted into efficient expression vectors (Examples 2 and 3).

The appropriate DNA fragments were identified and isolated by various methods. Thus, an immunologically active protein with a molecular weight of about 41 kD, which is also called p41 protein hereinafter, was prepared by means of the polymerase chain reaction (PCR) and specific primers whose sequences were prepared by synthesis (Example 2).

In addition, a gene bank of the *B. burgdorferi* genome was constructed and was screened using monoclonal antibodies for the direct expression of immunologically active proteins.

In a corresponding way, proteins with molecular weights of about 100 kD and 31 kD were also cloned and sequenced.

Another method comprised purifying particular selected immunologically active proteins (antigens) from *B. burgdorferi* lysates and determining the amino-acid sequences of these antigens. Subsequently, oligodeoxynucleotides corresponding to the amino-acid sequence were synthesized and, by hybridization, those clones in the gene bank which have DNA sequences coding for the immunologically active proteins were identified. The two latter methods are explained in detail in Example 3.

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After characterization, sequencing and recloning of the genes into appropriate expression vectors, the antigens were expressed in *E. coli* cells and subsequently purified. A preferred purification method is described in Example 4.

The immunologically active proteins from *Borrelia burgdorferi* which have been prepared according to the invention can be used in assay kits which provide a surprisingly sensitive detection of antibodies against *B. burgdorferi* in various test fluids. One advantage of the immunologically active proteins prepared according to the invention is that the preparations consist only of the required protein and possibly those proteins which are attributable to degradation events and/or incomplete translation. These preparations contain no *B. burgdorferi* proteins which do not correspond to the protein produced by recombination because they have been prepared by genetic manipulation.

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The term "assay kits" means a set of assay reagents which makes it possible to detect particular antibodies. The principles on which assay kits are based have been described in "Immunoassays for the 80s" (1981) by A. Voller et al., published by MTP Press Ltd., Falcon House, Lancaster, England. The assay reagents display as principal component the antigen(s)

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and, where appropriate, specific, preferably monoclonal, antibodies.

10 The assay kits according to the invention for detecting antibodies against *Borrelia burgdorferi* contain at least one immunologically active protein which is available without contamination by other proteins from the *Borrelia burgdorferi* strain. This immunologically active protein acts as antigen and reacts with the antibodies present in the test fluid. Assay kits according to the invention preferably have two to four immunologically active proteins which are available without contamination by other proteins from *B. burgdorferi*. The assay kit furthermore contains an indicator component which makes the detection of the presence of complexes of antigen and antibody possible.

20 The assay kits according to the invention can be based on a variety of principles known per se. In principle, the antigen can carry a label, and the label can consist of a radioactive isotope or an enzyme which catalyzes a color reaction. It is likewise possible for the antigen to be bound to a solid support (microtiter plates or beads), and the indicator component can comprise an antibody which is directed against antibodies and carries a label, and the label can comprise a radioactive isotope or an enzyme which catalyzes a color reaction.

30 The assay kit preferred for the purposes of the present invention is the so-called ELISA (enzyme-linked immunosorbent assay). One embodiment thereof is described in detail in Example 5. The results of this example show that it was surprisingly possible to achieve a very high specificity of the assay kit by using only one immunologically active protein according to the invention. Furthermore, the assay kits according to the invention surprisingly make possible a differentiation

correlated with the stage of the disease. The combined use of a plurality of antigens in one assay kit makes it possible to detect antibodies against *Borrelia burgdorferi* even in cases in which the symptoms of the disease have not yet become clinically manifest. It is likewise possible to diagnose infections with *B. burgdorferi* in which the patient experiences only a subclinical infection. The information which can be obtained from the assay kits according to the invention is particularly important in cases in which it has been possible to find a tick bite but it is unclear whether an infection with a *Borrelia* strain is present.

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Combined use of a plurality of the immunologically active proteins is preferred for the purpose of the present invention. A combination of the proteins p41, pC, p17 and/or p100 is very particularly preferred. The use of the ELISA assay kit preferred according to the invention also makes possible a differentiation with regard to the nature of the antibodies. If, for example, IgM antibodies are to be detected, the so-called μ capture assay can be employed, in which antibodies directed against IgM antibodies are bound to the solid phase. After the assay plates have been incubated with the fluid to be tested, the IgM antibodies present in the test fluid are bound to the solid phase. It is then possible, after saturation of non-specific bindings, to add an immunologically active protein of the present invention. This antigen is then detected by an indicator molecule. In this case the antigen can be biotinylated, and subsequently avidin which has covalently bonded peroxidase is added. The peroxidase then catalyzes a reaction which leads to color formation.

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Another possibility comprises adding monoclonal antibodies, which are specific for the antigen and are biotinylated, to the complex of support/anti-IgM

antibody/antibody to be detected/antigen according to the invention. Biotinylation is described, for example, in Monoklonale Antikörper [Monoclonal antibodies] (1985) Springer Verlag, J.H. Peters et al. Detection of the complex is effected therein by adding avidin to which an enzyme catalyzing a color reaction is coupled.

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Another embodiment of the present invention comprises detecting IgM by indirect ELISA. This entails the antigens according to the invention being bound to microtiter plates, incubated with the fluid to be detected and, after washing, the immune complexes being detected by means of anti- μ conjugate.

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Another aspect of the present invention comprises a generation of monoclonal antibodies which are directed against the immunologically active proteins of *Borrelia burgdorferi*. The preparation of monoclonal antibodies of this type is explained in detail in Example 6. It is possible to use monoclonal antibodies of this type as reagents for direct pathogen detection. However, monoclonal antibodies can also be coupled to the solid phase of a microtiter plate. The immunologically active proteins (antigens) are added and then immobilized by antibody-antigen binding to the microtiter plate. The test fluid (which can be, for example, serum or CSF) is subsequently added. The antibodies present in the test fluid then bind to the antigen and can be detected with the aid of an indicator component.

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Furthermore, the monoclonal antibodies can be used very satisfactorily for purifying immunologically active proteins (antigens). The advantage in this case is that the purification is particularly gentle. To do this, the monoclonal antibodies are bound to a solid matrix. This solid matrix is preferably in the form of a column. The partially prepurified antigens are then mixed under physiological conditions with the

- 11 -

antibodies coupled to a solid matrix. After the matrix-antibody-antigen complex has been washed it is possible to elute the antigens. It is normal to use for this high salt concentrations or buffers with a pH which makes the elution possible.

In another aspect of the present invention, DNA sequences which correspond in whole or in part to the amino-acid sequence of the immunologically active proteins are provided. These DNA sequences can preferably
10 be used to detect *Borrelia* strains in test material by hybridization. To do this, an oligonucleotide which partly corresponds to the DNA sequence is prepared. This oligonucleotide is radioactively labeled. On the other hand, the DNA from the test material is bound to a suitable filter, preferably nitrocellulose, and subsequently hybridized with the radioactively labeled oligonucleotide. It is likewise possible to use the DNA sequences according to the invention for in situ hybridization for direct detection of *B. burgdorferi* in
20 infected tissue. In place of the chemically synthesized oligonucleotides it is also possible for appropriate DNA fragments to be replicated in bacteria and subsequently cut out of the vectors with the aid of restriction endonucleases. After isolation of these DNA fragments they can be radioactively labeled and used as described above for the hybridization.

Another aspect of the present invention comprises the possibility of using the immunologically active proteins (antigens) according to the invention from
30 *Borrelia burgdorferi* for preparing vaccines for protection against infections by *Borrelia* bacteria, especially *Borrelia burgdorferi* strains. To do this,

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the antigens according to the invention are prepared in pure form. They are subsequently administered, singly or in combination with or without an agent stimulating the immune response, to the person to be immunized. This stimulates the formation of specific antibodies against *Borrelia burgdorferi* strains.

The proteins, DNA sequences and monoclonal antibodies according to the invention can be used in various areas. Thus, the assay kits according to the invention can also be used to detect *B. burgdorferi* infections in livestock, and the proteins can also be used for immunizing livestock, especially valuable livestock.

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To the extent that the present invention relates to proteins from *Borrelia burgdorferi*, these can also be protein fragments which have only a partial sequence of the complete amino-acid sequence. Partial sequences of this type usually have at least 10 amino acids and preferably at least 15 amino acids.

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However, the protein fragments are normally larger. Thus, for example, it has been found with the protein with a molecular weight of about 41 kD that deletion of about 20 to 25 amino acids at the N terminus of the protein leads to a protein which has an increased specificity. The reason for this might be that a so-called common epitope is deleted and specific epitopes remain. The use of proteins with deletions of this type is particularly preferred in this connection.

Proteins with a molecular weight of about 22 kD or 100 kD are particularly preferred for the purpose of the present invention. These proteins can also derive from other *Borrelia burgdorferi* strains.

The preferred embodiments of the present invention are explained in detail by means of the following tables, figures and examples.

Example 1:

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Determination of the immunorelevant and genus-specific *Borrelia* proteins

Specific, commonly occurring serum antibodies, which are directed against particular individual *B. burgdorferi* antigens, show minimum cross-reactivity with proteins of related pathogens and, in addition, permit correlation with the individual stages of the disease of Lyme borreliosis, were sought.

10 The Western blot was used to search for commonly recognized antigens. To do this, a bacterial extract of *B. burgdorferi* (PKo strain) (Preac-Mursic, V.; Wilske, B.; Schierz, G. (1986): European *Borrelia burgdorferi* isolated from humans and ticks - culture conditions and antibiotic susceptibility. Zbl. Bakt. Hyg. A 163, 112-118) was pelleted, resuspended in PBS/NaCl and treated with ultrasound and then fractionated by SDS polyacrylamide gel electrophoresis (Laemmli, U.K. (1970): Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685).

20 The gels consisted of a collecting gel with pockets for the samples and a separating gel. The separating gels had the following composition: 15% acrylamide (Bio-Rad), 0.026% diallyltartardiamide (DATD, Bio-Rad) per percent acrylamide, 0.15% SDS, 375 mM Tris-HCl pH 8.5, 0.14 mM ammonium peroxodisulfate (AMPER, Bio-Rad) and 0.035% N,N,N',N'-tetramethylethylenediamine (TEMED, Bio-Rad). AMPER and TEMED acted in this case as the radical initiators for the polymerization. 2-4 h after the polymerization, the collecting gel (3.1% acrylamide, 0.08% diallyltartardiamide, 0.1% SDS, 125 mM Tris-HCl pH 7.0, 3 mM AMPER and 0.05% TEMED) was poured
30 over the separating gel and provided with a Teflon comb. The anode and cathode chamber were filled with identical buffer solution: 25 mM tris base, 192 mM glycine and 0.1% SDS, pH 8.5.

In each case 20 μ l of sample in lysis buffer (3% sucrose, 2% SDS, 5% β -mercaptoethanol, 20 mM Tris-HCl pH 7.0, bromophenol blue; heated at 100°C for 5 min) were loaded per pocket. The electrophoresis was carried out at room temperature overnight with a constant

current of 6 mA for gels 20 x 15 cm in size. The gels were subsequently transferred to nitrocellulose (NC).

For the protein transfer, the gel with the nitrocellulose lying on it was placed between Whatman 3MM filter paper, conductive foam 1 cm thick and two carbon plates which conducted the current via platinum electrodes. Filter paper, foam and nitrocellulose were thoroughly impregnated with blot buffer (192 mM glycine, 25 mM tris base, 20% methanol, pH 8.5).
10 Transfer took place at 2 mA/cm² for 2 h. Free binding sites on the nitrocellulose were saturated for 1 h at 37°C with Cohen buffer (1 mg/ml Ficoll 400, 1 mg/ml polyvinylpyrrolidone, 16 mg/ml bovine serum albumin, 0.1% NP 40, 0.05% Bacto gelatin in sodium borate buffer pH 8.2); (Cohen G.H., Dietzschold, B., Ponce de Leon, M., Long, D., Golub, E., Varrichio, A., Pereira, L. and Eisenberg, R.J.: Localisation and synthesis of an antigenic determinant of Herpes simplex virus glycoprotein D that stimulates the production of
20 neutralizing antibodies. J. Virol. 49 (1984) 4183-4187). The blot was incubated with the patients' sera (1:100 dilution in 154 mM NaCl and 10 mM Tris-HCl pH 7.5) at room temperature overnight and with shaking. After the serum incubation, the blot was washed with TTBS (50 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.01% TweenTM 20) four times for 15 minutes each time. The blot was then incubated with peroxidase-coupled anti-human immunoglobulin (DAKO, Hamburg, 1:1000 dilution in 154 mM NaCl and 10 mM Tris-HCl, pH 7.5) at room
30 temperature for 2 h. The blot was washed several times with TTBS and then stained with 0.5 mg/ml diaminobenzidine and 0.01% hydrogen peroxide in 50 mM Tris-HCl pH 7.5. The staining was subsequently stopped with 1 N sulfuric acid, the blot was washed with water until free of acid and was dried between filter paper.

A selection of the reaction patterns of various sera with the Western blot strips is shown in Figures 1, 2a and b.

The following proteins proved to be immunodominant: p17 (17kDa), pC (22kDa), p41 (41kDa) and p100 (100kDa with variation in size in different *B. burgdorferi* isolates). Apart from p41, the biological functions of these antigens are unknown; p41 is the flagellin protein (Barbour, A.G.S., Hayes, S.F., Heiland, R.A., Schrupf, M.E. and Tessier, S.L.: A *Borrelia* genus-specific monoclonal antibody binds to a flagellar epitope. *Infect. Immun.* 52 (1986) 549-554).

10 These analyses, which were carried out with a relatively large number of patients' sera from the various stages of the disease, provided evidence that not all *B. burgdorferi* infections are always detected with a single antigen. It emerged, in particular in the case of sera with IgM antibodies (recent infection), that a protein (pC) in the 22 kD region is particularly often
20 recognized besides the flagellin (p41). However, simultaneous occurrence of both antibodies was not inevitable. It was possible to find sera which had only antibodies against p41 or only antibodies against the
✓ pC protein (Fig. 1 and 2a, Western blots). Detection of intrathecally formed antibodies in the CSF is of great importance in neuroborreliosis. IgG Western blots on
12 CSF/serum pairs from patients with Bannwarth's lymphocytic meningoradiculitis showed in all cases a local intrathecal immune response to p41. In the late
30 stage, besides IgG antibodies against the flagellin, particularly found were antibodies against proteins in the 100 kD region (p100) and in the 17 kD region (p17) which were undetectable or only rarely detectable in the early stages. Thus, antibody reactivities with the p17 and p100 proteins are good markers for the attain-
✓ ment of stage III (Fig. 2b, Western Blot).

Improved standardization of the assays can be achieved with the aid of these four antigens.

The proteins p42, pC and p17 additionally show only a slight cross-reactivity with other bacterial strains, and the protein p100 proved to be a genus-specific protein with *B. burgdorferi*-specific epitopes. Tab. 2 (reactivity of immune sera against various bacterial pathogens with proteins from *B. burgdorferi*) summarizes the cross-reactivity of sera against various related pathogens with *B. burgdorferi* antigens according to Western blot analysis. It emerged from attempts to
10 purify the four proteins (p41, pC, p17, p100) from *B. burgdorferi* extracts that large amounts of starting material are required. It was particularly difficult to purify p100, which is under-represented in the complete extract. Since cultivation is elaborate and costly it was necessary to look for possible ways of preparing these antigens by genetic manipulation. Western blot analysis of patients' sera has shown that virtually complete identification of all positive sera is possible with a combination of p41, pC, p17 and p100
20 produced by genetic manipulation as antigen and, furthermore, there is a correlation with the stage of the disease.

Example 2:

Production of p41 (flagellin) from *B. Burgdorferi* in *Escherichia coli* by genetic manipulation

The p41 coding region was obtained from a complete *B. burgdorferi* (DSM No. 5662 P/Ko2/85) DNA preparation by means of DNA amplification by a polymerase chain reaction (PCR). The sequence obtained in this way was
30 subsequently placed under the control of inducible promoters and, after transfection into *E. coli*, expression was induced (Maniatis, T.; Fritsch, E.F.; Sambrook, J. (1982) Molecular cloning. Cold Spring Harbor).

For this purpose, the *B. burgdorferi* cells were cultivated for 2 weeks at 37°C in 2 l of modified Barbour-

Stoenner-Kelly (BSK) medium (Preac-Mursic, V.,; Wilske, B.; Schierz, G. (1986): European *Borrelia burgdorferi* isolated from humans and ticks - culture conditions and antibiotic susceptibility. Zbl. Bakt. Hyg. A 163, 112-118), pelleted at 6000 rpm, washed in TEN buffer (10 mM Tris-HCl pH 7.6; 1 mM EDTA; 10 mM NaCl) and resuspended in 20 ml of lysozyme buffer (20% sucrose, 50 mM Tris-HCl pH 7.6, 50 mM EDTA, 5 mg/ml lysozyme). After incubation at 37°C for 30 min, the protoplasts produced by the action of lysozyme on the cell wall were lysed by adding 1 ml of 25% SDS (sodium dodecylsulfate). After a further 10 min, 4 ml of a 5 M NaCl solution were added. Protein was denatured by adding an equal volume of TE-saturated (TE: 10 mM Tris/HCl, pH 7.8, 1 mM EDTA) phenol. The phases were separated by centrifugation at 4°C and 6500 rpm for 5 min. Using a pipette with a wide opening (to avoid shear forces) the upper DNA-containing aqueous phase was cautiously transferred into a fresh tube and subsequently extracted once again with the same volume of phenol/chloroform/isoamyl alcohol (1:1:0.04). After separation, the upper aqueous phase was again cautiously transferred into a new tube, and the DNA was precipitated with twice the volume of ethanol. After about 5 min, the DNA which has precipitated out in the form of long threads is removed by winding onto a glass rod and is transferred into a 70% ethanol solution for washing. The DNA bound by adhesion to the glass rod was subsequently stored at room temperature for 2 h in order to bring about evaporation of the ethanol, and was then transferred into 4 ml of TEN buffer.

1 μ l portions of the complete *B. burgdorferi* DNA obtained in this way were amplified in 100 μ l PCR mixtures.

The sequences chosen as specific primers for the polymerase-catalyzed amplification contained the information for the translational start and the 3' end of p41 (flagellin). The DNA sequences shown in Fig. 3 were

used for this. The two oligodeoxynucleotides were synthesized in 1 μ mol columns in a Milligen/Biosearch 8700 DNA synthesizer and, after cleavage with ammonia, roughly purified by ethanol precipitation and taken up in 400 μ l of H₂O in each case. 1 μ l portions of this oligodeoxynucleotide solution were employed for each PCR mixture; the buffers, nucleotides and the Taq polymerase originated from a commercially obtainable assay kit (Cetus/Perkin-Elmer, Überlingen) and were also used in accordance with the assay descriptions. The temperature conditions for the individual cycles were:

2 min denaturation at	94°C
2 min annealing at	45°C
4 min DNA synthesis at	73°C

50 cycles were carried out.

The mixtures from the PCRs were subsequently combined, and the DNA was precipitated, after adding NaCl in a final concentration of 0.2 M, with 2.5 times the amount of ethanol at -20°C for 5 h. After pelleting and washing in 70% ethanol, the DNA was dissolved in 200 μ l of H₂O and, after addition of appropriate buffers, cleaved with the restriction enzymes Bam HI and Pst I (Boehringer Mannheim) as stated by the manufacturer.

Fractionation by gel electrophoresis in a 1.5% agarose gel was followed by isolation of the amplified DNA fragment (about 1000 bp) and insertion into a pUC8 vector (Pharmacia) cut with BamHI and PstI (Vieira, J.; Messing, J. (1982): The pUC plasmids, and M13mp7-derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene 19, 259-268), using 0.25 μ g of the vector, 0.5 μ g of the p41 fragment and 2U of T4 DNA ligase with buffer as specified by the manufacturer (Boehringer Mannheim).

The ligated DNA fragments were transformed into the E. coli strain JM 109 (Pharmacia) (Yanisch-Perron, C.; Vieira, J.; Messing, J. (1985): Improved M13 phage cloning vectors and host strains: nucleotide sequences

pUC8 for the production of p41. Expression of an authentic p41 which has no foreign amino acids fused on is possible by cleaving the p41-encoding region at the translation start with BspHI (TC ATG A) and PstI (at the 3' end) and inserting the fragment into the NcoI site (CC ATG G) and PstI site of a so-called ATG vector.

The clone pUC8ly17 was used for the methods indicated hereinafter.

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Example 3:

Production of pC, OspA and p100 in E. coli from B. burgdorferi gene banks

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To prepare B. burgdorferi-specific DNA sequences, a chromosomal gene bank was set up in E. coli. It was possible with the aid of suitable methods such as immunoscreening or hybridization with selected oligonucleotides to identify in this gene bank E. coli clones which contained corresponding B. burgdorferi-specific DNA sequences. A restriction enzyme map was constructed after restriction enzyme analysis. It was possible to use this to transfer the DNA sequences which were sought specifically into expression vectors and to carry out sequencing thereof. The specific procedures for this were as follows: to isolate B. burgdorferi (DSM No. 5662) DNA (chromosomal DNA and plasmid DNA), the cells were cultivated as described in Example 2. After centrifugation at 12,000 rpm for 20 minutes, the cells were washed and resuspended in SET buffer (20% sucrose, 50 mM Tris-HCl pH 7.6; 50 mM EDTA). The cell wall was partially cleaved by adding 15 mg/5 ml lysozyme for 20 minutes. The protoplasts from the cells were then lysed by adding SDS (n-dodecyl-sulfate sodium salt) final concentration 1%. After 20 minutes at 37°C, proteinase K (final concentration 1 mg/ml) was added for 1 hour twice, and the DNA-containing solution was adjusted to 100 mM NaCl

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with TEN buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 300 mM NaCl). One phenol extraction and two further phenol/chloroform/iso-amyl alcohol extractions (phenol:chloroform in the ratio 1:1; chloroform:iso-amyl alcohol in the ratio 24:1) were carried out. The supernatant extracted in this way was mixed with 2.5 vol. of 95% ethanol, and the DNA was precipitated at -20°C. It was possible to obtain the DNA by winding the precipitated threads onto a glass rod and to wash it in 70% ethanol. After brief drying in a desiccator, the DNA was taken up in TE buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA) which contained RNase (20 µg/ml). The DNA prepared in this way was used for the subsequent steps.

B. burgdorferi DNA was incubated with the restriction enzyme *Sau* 3A (Boehringer, Mannheim) as stated by the manufacturer. Partial cleavage of the DNA was achieved by choosing suitable enzyme dilutions and the time the enzyme acted thereon. Partially cleaved DNA obtained in this way was ligated with vector DNA (pUC18 or other suitable vector DNA) which had been restricted with *Bam*H I and dephosphorylated by treatment with alkaline phosphatase. T4 DNA ligase (Boehringer Mannheim) was employed as specified by the manufacturer for this. 0.2-0.5 µg/µl complete DNA was employed per transformation mixture. *E. coli* JM 109 (or other suitable *E. coli* strains) were transformed with the ligated DNA by the protocol of Hanahan (Hanahan, D. (1985): *Techniques of Transformation of Escherichia coli*, pp. 109-135. In: D.M. Glover (ed.) *DNA cloning*, Vol. 1. A practical approach. IRL Press, Oxford) or as described by Maniatis et al. (Maniatis, T. (1982): *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York). Recombinant *E. coli* clones were selected and cultivated on LB medium (10 g of tryptone, Difco, 5 g of yeast extract, Difco, 5 g of NaCl, Merck) which contained 100 µg/ml ampicillin (or another suitable antibiotic). The colony

pattern was transferred identically to LB plates, and colony replicas on nitrocellulose were produced. The cells in these colony replicas were lysed in different ways on the filter depending on the screening method used. When mono- or polyclonal sera (immunoscreening) were used to detect *B. burgdorferi*-specific gene products induced by the DNA inserted by recombination, the cells were lysed over saturated chloroform vapor for 15 min. After saturation of the filter treated in this way with a skimmed milk solution for 2 hours, the filters were incubated with the various sera overnight, washed several times with TTBS buffer (see above) and incubated with the second peroxidase-conjugated antibody (Dako, Hamburg) for 2 hours. Renewed washing with TTBS buffer served to reduce non-specifically bound peroxidase-conjugated antibodies. It was possible to identify positive, that is to say *B. burgdorferi* antigen-producing *E. coli* clones by enzymatic conversion of the substrates diaminobenzidine (Sigma-Chemie, Munich) and H_2O_2 into an insoluble brown pigment. The positive *E. Coli* clones identified in this way were inoculated from the initial plate and analyzed. When specific oligonucleotides were used for the hybridization and thus for the detection of specific *B. burgdorferi* antigen sequences (screening by hybridization), the cells underwent alkaline lysis on the nitrocellulose filter (Schleicher & Schuell) (by wetting the filters with 0.5 M NaOH, 1.5 M NaCl for 5 minutes). After neutralization (by wetting the filters in 1.5 M NaCl, 0.5 M Tris-HCl pH 8.0 for 5 minutes), the filters with the denatured DNA were wetted with 2x SSPE (20x SSPE: 3.6 M NaCl, 200 mM NaH_2PO_4 , pH 7.4, 20 mM EDTA, pH 7.4) and dried. The DNA was immobilized by baking the filters at 80°C for 2 hours. The filters treated in this way were then employed for the hybridization. The hybridization was carried out using radioactive (^{32}P) and non-radioactive (for example digoxigenin, Boehringer Mannheim) as

detection methods. The labeling methods for this were known (Maniatis, T. (1982): Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor) or recommended by the manufacturer (Boehringer Mannheim) (^{32}P labeling with ^{32}P -gamma-ATP and kinase reaction or digoxigenin labeling with Dig-11-UTP and terminal transferase reaction). A restriction enzyme analysis was drawn up for positive E. coli clones and, with this information, expression of the antigen-encoding DNA sequences in suitable vectors, and sequencing thereof, were carried out.

10

The hybridization probes employed at the start were synthetic oligodeoxynucleotides whose sequence had been selected on the basis of p100 and pC amino-acid sequences.

The procedure for this was as follows:

The two proteins were partially purified from lysates of B. burgdorferi by extraction with n-octyl β -D-thio-glucopyranoside and further fractionated by SDS-polyacrylamide gel electrophoresis. The antigens were subsequently transferred by Western blotting to a glass fiber matrix, and the appropriate pieces with the B. burgdorferi proteins were cut out. p100 then underwent partial N-terminal sequencing, and the first 22 amino acids of the amino terminus were determined (this method of micro-sequencing is described in: Eckerskorn, C., Mewes, W., Goretzki, H. and Lottspeich, F.: A new siliconized fiber as support for protein-chemical analysis of electroblotted proteins. Eur. J. Biochem. 176 (1988) 509-519). In the case of pC, direct partial sequencing was not possible since the N terminus is not directly amenable to sequencing, that is to say that it is possible that myristylation or similar modifications are present. For this reason, this protein was cleaved with trypsin, the fragments were fractionated by HPLC, and two of them were then partially sequenced. The oligodeoxynucleotide sequences specified hereinafter were then derived from the amino-

20

30

acid sequences obtained in this way. Since in most cases there are several codon options for an amino acid, it was also necessary for the base variations and the appropriate sites on the oligonucleotide to be taken into account and incorporated during the synthesis in equimolar ratios.

p100-p1 - p100 - amino-acid sequence:

Glu Leu Asp Lys Glu Lys Leu Lys Asp Phe Val Asn Leu Asp
Leu Glu Phe Val Asn Thr

10 p-100-oligodeoxynucleotide sequence, the bases indicated in parentheses and separated by ";" were incorporated during the synthesis (in a Milligen/Biosearch 8700 DNA synthesizer) in equimolar ratios:

GA(G;A) (C;T)T(G;T;A) GA(C;T) AA(G;A) GA(G;A) AA(G;A)
(C;T)T(G;T;A) AA(G;A) GA(C;T) TT(C;T) GT(T;A) AA(C;T)
(C;T)T(G;T;A) GA(C;T) (C;T)A(G;T;A) GA(G;A) TT(C;T) GT(T;A)
AA(C;T) TA(C;T) A

20 The oligodeoxynucleotide sequence was used as probe and hybridized with the clones containing the *B. burgdorferi* DNA. Subcloning results in a clone which contains the gene for p100. The following coding DNA sequence of p100 (5' end) of the strain PKo was found for a length of 346 base pairs:

30 5' ATG AAA AAA ATG TTA CTA ATC TTT AGT TTT TTT CTT GTT
TTT TTA AAT GGA TTT CCT CTT AAT GCA AGG GAA GTT GAT AAG GAA
AAA TTA AAG GAC TTT GTT AAT ATG GAT CTT GAA TTT GTT AAT TAC
AAG GGT CCT TAT GAT TCT ACA AAT ACA TAT GAA CAA ATA GTA GGT
ATT GGG GAG TTT TTA GCA AGG CCG TTG ATC AAT TCC AAT AGT AAG
TCA AGT TAT TAT GGT AAA TAT TTT GTT AAT AGA TTT ATT GAC GAT
CAA GAT AAA AAA GCA AGT GTT GAT ATT TTT TCT ATT GGT AGT AAG
TCA GAG CTT GAT AGT ATA TTA AAT CTA AGA AGA ATT C... 3'

The following amino-acid sequence was found after complete cloning:

	Met	Lys	Lys	Met	Leu	Leu	Ile	Phe	Ser	Phe	Phe	Leu	Val	Phe	Leu
	Asn	Gly	Phe	Pro	Leu	Asn	Ala	Arg	Glu	Val	Asp	Lys	Glu	Lys	Leu
	Lys	Asp	Phe	Val	Asn	Met	Asp	Leu	Glu	Phe	Val	Asn	Tyr	Lys	Gly
	Pro	Tyr	Asp	Ser	Thr	Asn	Thr	Tyr	Glu	Gln	Ile	Val	Gly	Ile	Gly
	Glu	Phe	Leu	Ala	Arg	Pro	Leu	Ile	Asn	Ser	Asn	Ser	Asn	Ser	Ser
	Tyr	Tyr	Gly	Lys	Tyr	Phe	Val	Asn	Arg	Phe	Ile	Asp	Asp	Gln	Asp
10	Lys	Lys	Ala	Ser	Val	Asp	Ile	Phe	Ser	Ile	Gly	Ser	Lys	Ser	Glu
	Leu	Asp	Ser	Ile	Leu	Asn	Leu	Arg	Arg	Ile	Leu	Thr	Gly	Tyr	Leu
	Ile	Lys	Ser	Phe	Asp	Tyr	Glu	Arg	Ser	Ser	Ala	Glu	Leu	Ile	Ala
	Lys	Ala	Ile	Thr	Ile	Tyr	Asn	Ala	Val	Tyr	Arg	Gly	Asp	Leu	Asp
	Tyr	Tyr	Lys	Glu	Phe	Tyr	Ile	Glu	Ala	Ser	Leu	Lys	Ser	Leu	Thr
	Lys	Glu	Asn	Ala	Gly	Leu	Ser	Arg	Val	Tyr	Ser	Gln	Trp	Ala	Gly
	Lys	Thr	Gln	Ile	Phe	Ile	Pro	Leu	Lys	Lys	Asn	Ile	Leu	Ser	Gly
	Asn	Val	Glu	Ser	Asp	Ile	Asp	Ile	Asp	Ser	Leu	Val	Thr	Asp	Lys
	Val	Val	Ala	Ala	Leu	Leu	Ser	Glu	Asn	Glu	Ser	Gly	Val	Asn	Phe
	Ala	Arg	Asp	Ile	Thr	Asp	Ile	Gln	Gly	Glu	Thr	His	Lys	Ala	Asp
	Gln	Asp	Lys	Ile	Asp	Ile	Glu	Leu	Asp	Asn	Phe	His	Glu	Ser	Asp
20	Ser	Asn	Ile	Thr	Glu	Thr	Ile	Glu	Asn	Leu	Arg	Asp	Gln	Leu	Glu
	Lys	Ala	Thr	Asp	Glu	Glu	His	Lys	Lys	Glu	Ile	Glu	Ser	Gln	Val
	Asp	Ala	Lys	Lys	Lys	Gln	Lys	Glu	Glu	Leu	Asp	Lys	Lys	Ala	Ile
	Asp	Leu	Asp	Lys	Ala	Gln	Gln	Lys	Leu	Asp	Phe	Ala	Glu	Asp	Asn
	Leu	Asp	Ile	Gln	Arg	Asp	Thr	Val	Arg	Glu	Lys	Leu	Gln	Glu	Asn
	Ile	Asn	Glu	Thr	Asn	Lys	Glu	Lys	Asn	Leu	Pro	Lys	Pro	Gly	Asp
	Val	Ser	Ser	Pro	Lys	Val	Asp	Lys	Gln	Leu	Gln	Ile	Lys	Glu	Ser
	Leu	Glu	Asp	Leu	Gln	Glu	Gln	Leu	Lys	Glu	Ala	Ser	Asp	Glu	Asn
	Gln	Lys	Arg	Glu	Ile	Glu	Lys	Gln	Ile	Glu	Ile	Lys	Lys	Asn	Asp
	Glu	Glu	Leu	Phe	Lys	Asn	Lys	Asp	His	Lys	Ala	Leu	Asp	Leu	Lys
30	Gln	Glu	Leu	Asn	Ser	Lys	Ala	Ser	Ser	Lys	Glu	Lys	Ile	Glu	Gly
	Glu	Glu	Glu	Asp	Lys	Glu	Leu	Asp	Ser	Lys	Lys	Asn	Leu	Glu	Pro
	Val	Ser	Glu	Ala	Asp	Lys	Val	Asp	Lys	Ile	Ser	Lys	Ser	Asn	Asn
	Asn	Glu	Val	Ser	Lys	Leu	Ser	Pro	Leu	Asp	Glu	Pro	Ser	Tyr	Ser
	Asp	Ile	Asp	Ser	Lys	Glu	Gly	Val	Asp	Asn	Lys	Asp	Val	Asp	Leu
	Gln	Lys	Thr	Lys	Pro	Gln	Val	Glu	Ser	Gln	Pro	Thr	Ser	Leu	Asn
	Glu	Asp	Leu	Ile	Asp	Val	Ser	Ile	Asp	Ser	Ser	Asn	Pro	Val	Phe
	Leu	Glu	Val	Ile	Asp	Pro	Ile	Thr	Asn	Leu	Gly	Thr	Leu	Gln	Leu
	Ile	Asp	Leu	Asn	Thr	Gly	Val	Arg	Leu	Lys	Glu	Ser	Ala	Gln	Gln
	Gly	Ile	Gln	Arg	Tyr	Gly	Ile	Tyr	Glu	Arg	Glu	Lys	Asp	Leu	Val
40	Val	Ile	Lys	Ile	Asp	Ser	Gly	Lys	Ala	Lys	Leu	Gln	Ile	Leu	Asp
	Lys	Leu	Glu	Asn	Leu	Lys	Val	Ile	Ser	Glu	Ser	Asn	Phe	Glu	Ile
	Asn	Lys	Asn	Ser	Ser	Leu	Tyr	Val	Asp	Ser	Arg	Met	Ile	Leu	Val
	Val	Val	Lys	Asp	Asp	Ser	Asn	Ala	Trp	Arg	Leu	Ala	Lys	Phe	Ser
	Pro	Lys	Asn	Leu	Asp	Glu	Phe	Ile	Leu	Ser	Glu	Asn	Lys	Ile	Leu
	Pro	Phe	Thr	Ser	Phe	Ala	Val	Arg	Lys	Asn	Phe	Ile	Tyr	Leu	Gln
	Asp	Glu	Leu	Lys	Ser	Leu	Val	Thr	Leu	Asp	Val	Asn	Thr	Leu	Lys
	Lys	Val	Lys												

Amino-acid sequence of the p100 protein

In an analogous manner, using pC amino-acid sequences:

p1: Lys Ile Thr Asp Ser Asn Ala Thr Val Leu Ala Val Lys

p2: Asp Leu Phe Glu Ser Val Glu Gly Leu Leu Lys

the corresponding oligodeoxynucleotide sequences were synthesized:

pC-p1 oligodeoxynucleotide sequence:

AA(G;A) AT(T;A) AC(A;T) GA(T;C) (A;T)C(A;T) AA(T;C)

GC(A;T) AC(A;T) GT(A;T) (T;C)T(G;A;T) GC(A;T) GT(A;T)

AA(A;G) A

10 pC-p2 oligodeoxynucleotide sequence:

GA(T;C) (C;T)T(G;A;T) TT(T;C) GA(G;A) (T;A)C(A;T)

GT(A;T) GA(G;A) GG(A;T;C) (T;C)T(G;A;T) (T;C)T(G;A;T)

AA(A;G) A

After suitable clones have been found by hybridization and subcloning of the required gene it was possible to determine the following coding DNA sequence of pC of the strain PKo for a length of 639 base pairs:

5' ATG AAA AAG AAT ACA TTA AGT GCG ATA TTA ATG ACT TTA
TTT TTA TTT ATA TCT TGT AAT AAT TCA GGG AAG GTG GGG ATT CTG
20 CAT CTA CTA ATC CTG CTG ACG AGT CTT GCG AAA GGG CCT AAT CTT
ACA GAA ATA AGC AAA AAA ATT ACA GAT TCT AAT GCA TTT GTA CTT
GCT GTT AAA GAA GTT GAG ACT TTG GTT TTA TCT ATA GAT GAA CTT
GCT AAG AAA GCT ATT GGT CAA AAA ATA GAC AAT AAT AAT GGT TTA
GCT GCT TTA AAT AAT CAG AAT GGA TCG TTG TTA GCA GGA GCC TAT
GCA ATA TCA ACC CTA ATA ACA GAA AAA TTG AGT AAA TTG AAA AAT
TTA GAA GAA TTA AAG ACA GAA ATT GCA AAG GCT AAG AAA TGT TCC
GAA GAA TTT ACT AAT AAA CTA AAA AGT GGT CAT GCA GAT CTT GGC
AAA CAG GAT GCT ACC GAT GAT CAT GCA AAA GCA GCT ATT TTA AAA
ACA CAT GCA ACT ACC GAT AAA GGT GCT AAA GAA TTT AAA GAT TTA
30 TTT GAA TCA GTA GAA GGT TTG TTA AAA GCA GCT CAA GTA GCA CTA
ACT AAT TCA GTT AAA GAA CTT ACA AGT CCT GTT GTA GCA GAA AGT
CCA AAA AAA CCT TAA 3'

The protein pC has the following sequences for a length of 212 amino acids:

10 Met Lys Lys Asn Thr Leu Thr Ala Ile Leu Met Thr Leu Phe Leu
Phe Ile Ser Cys Asn Asn Ser Gly Lys Val Gly Ile Leu Thr Ser
Thr Asn Pro Ala Asp Glu Ser Ala Lys Gly Pro Asn Leu Thr Glu
Ile Ser Lys Lys Ile Thr Asp Ser Asn Ala Phe Val Leu Ala Val
Lys Glu Val Glu Thr Leu Val Leu Ser Ile Asp Glu Leu Ala Lys
Lys Ala Ile Gly Gln Lys Ile Asp Asn Asn Asn Gly Leu Ala Ala
Leu Asn Asn Gln Asn Gly Ser Leu Leu Ala Gly Ala Tyr Ala Ile
Ser Thr Leu Ile Thr Glu Lys Leu Ser Lys Leu Lys Asn Leu Glu
Glu Leu Lys Thr Glu Ile Ala Lys Ala Lys Lys Cys Ser Glu Glu
Phe Thr Asn Lys Leu Lys Ser Gly His Ala Asp Leu Gly Lys Gln
Asp Ala Thr Asp Asp His Ala Lys Ala Ala Ile Leu Lys Thr His
Ala Thr Thr Asp Lys Gly Ala Lys Glu Phe Lys Asp Leu Phe Glu
Ser Val Glu Gly Leu Leu Lys Ala Ala Gln Val Ala Leu Thr Asn
Ser Val Lys Glu Leu Thr Ser Pro Val Val Ala Glu Ser Pro Lys
Lys Pro

Amino-acid sequence of the pC protein - 22kD -

20 In a corresponding way, a part of the coding DNA
sequence of OspA (5' end) of the strain PKo was also
determined for a length of 680 base pairs:

5' ATG AAA AAA TAT TTA TTG GGA ATA GGT CTA ATA TTA GCC
TTA ATA GCA TGC AAG CAA AAT GTT AGC AGC CTT GAT GAA AAA AAC
AGC GCT TCA GTA GAT TTG CCT GGT GAG ATG AAA GTT CTT GTA AGT
AAA GAA AAA GAC AAA GAC GGT AAG TAC AGT CTA AAG GCA ACA GTA
GAC AAG ATT GAG CTA AAA GGA ACT TCT GAT AAA GAC AAT GGT TCT
GGG GTG CTT GAA GGT ACA AAA GAT GAC AAA AGT AAA GCA AAA TTA
ACA ATT GCT GAC GAT CTA AGT AAA ACC ACA TTC GAA CTT TTC AAA
GAA GAT GGC AAA ACA TTA GTG TCA AGA AAA GTA AGT TCT AAA GAC
30 AAA ACA TCA ACA GAT GAA ATG TTC AAT GAA AAA GGT GAA TTG TCT
GCA AAA ACC ATG ACA AGA GAA AAT GGA ACC AAA CTT GAA TAT ACA
GAA ATG AAA AGC GAT GGA ACC GGA AAA GCT AAA GAA GTT TTA AAA
AAC TTT ACT CTT GAA GGA AAA GTA GCT AAT GAT AAA GTA ACA TTG
GAA GTA AAA GAA GGA ACC GTT ACT TTA AGT AAG GAA ATT GCA AAA
TCT GGA GAA GTA ACA GTT GCT CTT AAT GAC ACT AAC ACT ACT CAG
GCT ACT AAA AAA ACT GGC GCA TGG GAT TCA AAA ACT TCT ACT TTA
ACA ATT AGT GT...3'

After complete sequencing it was possible to determine the following amino-acid sequence for the 31 kD protein:

	Met	Lys	Lys	Tyr	Leu	Leu	Gly	Ile	Gly	Leu	Ile	Leu	Ala	Leu	Ile
	Ala	Cys	Lys	Gln	Asn	Val	Ser	Ser	Leu	Asp	Glu	Lys	Asn	Ser	Ala
	Ser	Val	Asp	Leu	Pro	Gly	Glu	Met	Lys	Val	Leu	Val	Ser	Lys	Glu
	Lys	Asp	Lys	Asp	Gly	Lys	Tyr	Ser	Leu	Lys	Ala	Thr	Val	Asp	Lys
	Ile	Glu	Leu	Lys	Gly	Thr	Ser	Asp	Lys	Asp	Asn	Gly	Ser	Gly	Val
	Leu	Glu	Gly	Thr	Lys	Asp	Asp	Lys	Ser	Lys	Ala	Lys	Leu	Thr	Ile
10	Ala	Asp	Asp	Leu	Ser	Lys	Thr	Thr	Phe	Glu	Leu	Phe	Lys	Glu	Asp
	Gly	Lys	Thr	Leu	Val	Ser	Arg	Lys	Val	Ser	Ser	Lys	Asp	Lys	Thr
	Ser	Thr	Asp	Glu	Met	Phe	Asn	Glu	Lys	Gly	Glu	Leu	Ser	Ala	Lys
	Thr	Met	Thr	Arg	Glu	Asn	Gly	Thr	Lys	Leu	Glu	Tyr	Thr	Glu	Met
	Lys	Ser	Asp	Gly	Thr	Gly	Lys	Ala	Lys	Glu	Val	Leu	Lys	Asn	Phe
	Thr	Leu	Glu	Gly	Lys	Val	Ala	Asn	Asp	Lys	Val	Thr	Leu	Glu	Val
	Lys	Glu	Gly	Thr	Val	Thr	Leu	Ser	Lys	Glu	Ile	Ala	Lys	Ser	Gly
	Glu	Val	Thr	Val	Ala	Leu	Asn	Asp	Thr	Asn	Thr	Thr	Gln	Ala	Thr
	Lys	Lys	Thr	Gly	Ala	Trp	Asp	Ser	Lys	Thr	Ser	Thr	Leu	Thr	Ile
	Ser	Val	Asn	Ser	Lys	Lys	Thr	Thr	Gln	Leu	Val	Phe	Thr	Lys	Gln
20	Asp	Thr	Ile	Thr	Val	Gln	Lys	Tyr	Asp	Ser	Ala	Gly	Thr	Asn	Leu
	Glu	Gly	Thr	Ala	Val	Glu	Ile	Lys	Thr	Leu	Asp	Glu	Leu	Lys	Asn
	Ala	Leu	Lys												

Amino-acid sequence of OspA (strain PKo)

Example 4:

Purification of the *B. burgdorferi* antigens produced by recombination

a) p41 (flagellin) as example

30 A 50 ml overnight culture of the clone pUC8ly17 described in Example 2 was added to 1.5 ml of fresh L broth medium and incubated, shaking vigorously, at 37°C. When an optical density of 0.7 was reached, the culture was induced with IPTG in a final concentration of 1 mM and incubated for a further 3 h. The bacteria were pelleted (6000 rpm, 10 min), resuspended in 300 ml of 20 mM Tris-HCl pH 8.0, 50 mM EDTA, 0.5 mg/ml lysozyme and placed in a water bath at 37°C for 45 min. Addition of NaCl in a final concentration of 150 mM and Triton-X-100 in a final concentration of 1% was followed by further incubation at 37°C for 45 min, and

the suspension was subsequently treated with ultrasound three times for 5 min each time. Insoluble constituents were pelleted at 9000 rpm for 30 min, resuspended in 20 mM Tris-HCl pH 8.0, 10 mM dithiothreitol and 1% octyl glucopyranoside (Sigma-Chemie, Munich) and stirred at room temperature for 1 h. After subsequent pelleting of insoluble constituents at 17,000 rpm for 30 min, the supernatant was cautiously decanted off.

10 The pellet was subsequently resuspended in 150 ml of 20 mM Tris-HCl pH 8.0, 8 M urea and 1% β mercaptoethanol by stirring for 2 h. Insoluble constituents were once again in this case removed by centrifugation at 17,000 rpm for 30 min, and the supernatant was pumped onto a DEAE SephacelTM column (Pharmacia, Freiburg) with a gel volume of 550 ml (diameter 3 cm, height 80 cm). The p41 antigen was eluted in an NaCl gradient from 0 to 800 mM in a total volume of 600 ml. The recombinant p41 is eluted at an NaCl concentration of about 0.25 M. The appropriate fractions were combined and further
20 purified by HPLC with a Mono QTM column (anion exchanger) (Fig. 4). An elution profile with the purified p41 in an NaCl gradient from 0 to 800 mM is shown in Fig. 5. The fractions which were positive for p41 here (according to Western blot analysis) were dialyzed against 20 mM Tris-HCl pH 8.0, 10 mM MgCl₂ and 0.1% β -mercaptoethanol, and subsequently used for the assays shown in Example 5. The yield typically to be expected from purification of p41 starting from 1 l of bacterial culture is 5 to 10 mg.

30 It was possible to determine the following amino-acid sequence after sequencing:

10 Met Arg Gly Ser Ile Met Ile Ile Asn His Asn Thr Ser Ala Ile
 Asn Ala Ser Arg Asn Asn Ala Ile Asn Ala Ala Asn Leu Ser Lys
 Thr Gln Glu Lys Leu Ser Ser Asn Tyr Arg Ile Asn Arg Ala Ser
 Asp Asp Ala Ala Gly Met Gly Val Ser Gly Lys Ile Asn Ala Gln
 Ile Arg Gly Leu Ser Gln Ala Ser Arg Asn Thr Ser Lys Ala Ile
 Asn Phe Ile Gln Thr Thr Glu Gly Asn Leu Asn Glu Val Glu Lys
 Val Leu Val Arg Met Lys Glu Leu Ala Val Gln Ser Gly Asn Gly
 Thr Tyr Ser Asp Ser Asp Arg Gly Ser Ile Gln Ile Glu Ile Glu
 Gln Leu Thr Asp Glu Ile Asn Arg Ile Ala Asp Gln Ala Gln Tyr
 20 Asn Gln Met His Met Leu Ser Asn Lys Ser Ala Ser Gln Asn Val
 Lys Thr Ala Glu Glu Leu Gly Met Gln Pro Ala Lys Ile Asn Thr
 Pro Ala Ser Leu Ser Gly Ser Gln Ala Ser Trp Thr Leu Arg Val
 His Val Gly Ala Asn Gln Asp Glu Ala Ile Ala Val Asn Ile Tyr
 Ser Ala Asn Val Ala Asn Leu Phe Ala Gly Glu Gly Ala Gln Ala
 Ala Gln Ala Ala Pro Val Gln Glu Gly Ala Gln Glu Glu Gly Ala
 Gln Gln Pro Thr Pro Ala Thr Ala Pro Thr Gln Gly Gly Val Asn
 Ser Pro Val Asn Val Thr Thr Thr Val Asp Ala Asn Thr Ser Leu
 Ala Lys Ile Glu Asn Ala Ile Arg Met Ile Ser Asp Gln Arg Ala
 30 Asn Leu Gly Ala Phe Gln Asn Arg Leu Glu Ser Ile Lys Asn Ser
 Thr Glu Tyr Ala Ile Glu Asn Leu Lys Ala Ser Tyr Ala Gln Ile
 Lys Asp Ala Thr Met Thr Asp Glu Val Val Ala Ala Thr Thr Asn
 Ser Ile Leu Thr Gln Ser Ala Met Ala Met Ile Ala Gln Ala Asn
 Gln Val Pro Gln Tyr Val Leu Ser Leu Leu Arg

Amino-acid sequence of the p41 protein

b) Purification of recombinant *Borrelia burgdorferi* pC antigen from *E. coli*

30 A clone which contains the gene for the pC antigen (pDS1PC5) is inoculated in 100 ml of L broth (containing 50 µg of ampicillin/ml), left to grow overnight and then transferred into 900 ml of L broth/ampicillin - 2x concentrated yeast extract/2 ml of glycerol - and, after about 1 h, induced with 2 mM IPTG and shaken for a further 2-3 h.

40 The pellet, after centrifugation at 8000 rpm for 10 min, is resuspended in 20 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mM DTE, 0.1 mM PMSF; 0.4 mg/ml lysozyme). Stirring at room temperature for 30 min is followed by addition of Triton-X 100 (final concentration 0.1-0.2%). Also added are 10 µl of Benzonase (Merck). Stirring at room temperature is

continued for a further 30 min. The suspension which is now clear is adjusted to 1 M NaCl with solid NaCl and stirred for a further 30 min-60 min (at 4°C).

10 After centrifugation at 4°C and 15,000 rpm for 30 min, the pC protein is quantitatively present in the supernatant. The pellet is discarded. The supernatant is dialyzed against 10 mM Tris-HCl, pH 8.0, changing the buffer several times. Centrifugation and/or filtration is followed by loading onto DEAE Sepharose™ (Pharmacia), the column being equilibrated with 10 mM Tris-HCl, pH 8.0. On elution with 0 M NaCl, the pC protein appears in the second peak of the flow-through. The first fractions can be discarded, and the remainder is collected and rechromatographed. The separating column is regenerated with 1 M NaCl and equilibrated in 10 mM Tris-HCl pH 8.0. The antigen obtained in this way can now be used in a suitable assay kit, for example an ELISA.

20 c) Purification of recombinant *Borrelia burgdorferi* OspA antigen from *E. coli*

A clone which contains the gene for the OspA antigen (pDS1OspA) is inoculated in 100 ml of L broth (containing 50 µg of ampicillin/ml) and cultured overnight. The culture broth is transferred into 900 ml of L broth/ampicillin - 2x concentrated yeast extract/2 ml glycerol - and, after about 1 h, induced with 2 mM IPTG and shaken for a further 2-3 h.

30 The cells are centrifuged at 6000 rpm for 5 min, and the pellet is resuspended in 20 ml of lysis buffer (50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mM DTE, 0.1 mM PMSF; 0.4 mg/ml lysozyme). Stirring at room temperature for 30 min is followed by addition of Triton™-X 100 (final concentration 0.5-1%). Also added are 10 µl of Benzonase (MERCK). This is followed by stirring at room

temperature for a further 30 min.

The suspension which is now clear is adjusted to 1 M NaCl with solid NaCl and stirred further (at 4°C). After centrifugation at 4°C and 15,000 rpm for 30 min, OspA is virtually quantitatively present in the pellet. The supernatant is discarded, and the pellet is resuspended in 2 M urea (with 50 mM Tris-HCl, pH 7.5, 2 mM EDTA, 0.1 mM DTE). OspA is now in the supernatant.

10 The supernatant is dialyzed in a dialysis tube against 5 mM MES (2-[N-morpholino]ethanesulfonic acid) buffer, pH 6.0, it being absolutely necessary to change the buffer several times. After centrifugation and filtration, the protein is loaded onto an S Sepharose fast-flow (Pharmacia) column. It is first washed with 0 M NaCl and then eluted with a gradient from 0 to 1 M NaCl. The OspA antigen elutes as a sharp peak at about 0.4 M NaCl. After dialysis against 10 mM Tris-HCl pH 7.5, the OspA antigen can be used in a suitable assay kit, for example an ELISA.

20 **Example 5:**

Use of *B. burgdorferi* antigens produced by recombination (p41 as example) in an ELISA

Owing to the high purity of the recombinant antigens produced, it is possible to carry out *B. burgdorferi*-specific assays which are machine-readable and can be carried out without great technical and personnel expenditure.

30 Microtiter plates were coated with 50 μ l of the purified p41 (concentration 0.5-5 μ g/l) per well. The plates were incubated by standard methods at 4°C overnight, washed, and the binding sites which were still free were saturated with 2% strength bovine serum albumin solution. Subsequently, 50 μ l of serum (dilution 1:200) were pipetted into each and incubated

at 37°C for 2 h, unbound portions were washed out and the bound immune complexes were detected with 50 µl of peroxidase-labeled anti-human IgG (dilution 1:1000) in each case. Another wash was followed by each of the wells being charged with 100 µl of ortho-phenylenediamine (concentration 0.1% in 0.1 M phosphate buffer pH 6.0 with 0.03% H₂O₂) as color reagent, and the staining was carried out in the dark and stopped with 100 µl of 1 N sulfuric acid after 10 min. The microtiter plate was evaluated in a photometer at 486 nm (Fig. 6).

In the example shown here, 7 positive and 8 negative anti-*B. burgdorferi* sera were tested. Three of the clinically confirmed Lyme-positive sera showed no reaction with p41 on Western blot strips with *B. burgdorferi* as antigen, that is to say were sera from the early stage of infection. These likewise showed only marginal reaction in an ELISA with the recombinant antigen. By contrast, normally p41-positive sera reacted very well, whereas Lyme-negative sera remained in the range below OD = 0.3.

Example 6:

Preparation of *B. Burgdorferi*-specific monoclonal antibodies

Female Balb/C mice were immunized intraperitoneally with *B. burgdorferi* (DSM No. 5662). The first immunization was carried out with complete Freund's adjuvant, and 2-5 further immunizations with incomplete Freund's adjuvant followed at intervals of 2 weeks. 2 weeks later, the antigen was administered without adjuvant, and 3 days later the mice were sacrificed and the spleen was removed.

The spleen lymphocytes were mixed with mouse myeloma cells (Ag8-653) in the ratio 1:1, sedimented and mixed with fusion solution (2.5 g of polyethylene glycol (PEG), 2.5 ml of RPMI-medium, 250 µl of DMSO): 1 min

addition of the fusion solution, incubation at 37°C for 90 sec. The cells were again sedimented, the PEG was removed, and culture medium (HAT medium) was added. Finally, the cell suspension was inoculated into microtiter plates which contained macrophages as feeder cells and was incubated. Hybridoma supernatants were subjected undiluted to an indirect immunofluorescence assay (IFA) (Wilske, B.; Schierz, G.; Preac-Mursic, V.; Weber, K.; Pfister, H.-W.; Einhäupl, K. (1984): Serological diagnosis of Erythema migrans disease and related disorders. Infection, 12, 331-337).

10

IFA-positive cell supernatants were subjected to Western blot analysis. Hybridomas which reacted in the Western blot were subcloned 4 times by limiting dilution, and their immunoglobulin class and IgG subclass were identified.

The following monoclonal antibodies (MAB) were obtained in this way:

1. MAB against p41:

20

(a) L41 1C11

This antibody reacted with all 30 assayed *B. burgdorferi* strains and with *Borrelia* of relapsing fever (apart from *B. hermsii*) but not with *Treponema*.

(b) L41 1D3

This antibody reacted with the majority (21 of 24) of the *B. burgdorferi* strains but not with the *Borrelia* of relapsing fever and *Treponema*.

30

2. MAB against p100 (L100 1D4):

This antibody reacted with all 30 assayed *B. burgdorferi* strains but not with the *Borrelia* of relapsing fever or *Treponema*.

3. MAB against pC (L22 1F8):

This MAB reacted with pC proteins from strains from skin and CSF strains, whereas the pC proteins of some but not all tick strains were negative.

4. MAB against OspA:

OspA is a major protein (30 kD region) of the outer membrane of most *B. burgdorferi* strains. OspA proteins of European *B. burgdorferi* strains are antigenetically heterogeneous and differ antigenetically from the American strains. The few OspA-negative strains have pC proteins.

(a) L32 2E7

10 In total, 29 of 32 strains reacted. The negative strains had no OspA protein. The 3 negative strains reacted with the pC-specific MAB L22 1F8.

(b) L32 1G3:

This MAB reacted with only 3 of 25 assayed strains.

The combination of MAB L32 2E7 and MAB L22 1F8 and the reaction with MAB L100 1D4 allows identification of *B. burgdorferi* *Borrelia* and *Treponema*. Reliable identification and differentiation of *B. burgdorferi* has not been possible with monoclonal antibodies available to date.

20

Example 7:

Determination of the amino-acid sequence of a protein with a molecular weight of about 22kD from another strain

The amino-acid sequence of a protein with a molecular weight of about 22kD was determined by the methods described in the previous examples. This protein was cloned from another *Borrelia* strain and was subsequently sequenced. This strain has been deposited at the ATCC under the number 35210 and is generally accessible. The following amino-acid sequence was determined in this case:

30

10

Met Lys Lys Asn Thr Leu Ser Ala Ile Leu Met Thr Leu Phe Leu
Phe Ile Ser Cys Asn Asn Ser Gly Lys Asp Gly Asn Thr Ser Ala
Asn Ser Ala Asp Glu Ser Val Lys Gly Pro Asn Leu Thr Glu Ile
Ser Lys Lys Ile Thr Asp Ser Asn Ala Val Leu Leu Ala Val Lys
Glu Val Glu Ala Leu Leu Ser Ser Ile Asp Glu Ile Ala Ala Lys
Ala Ile Gly Lys Lys Ile His Gln Asn Asn Gly Leu Asp Thr Glu
Asn Asn His Asn Gly Ser Leu Leu Ala Gly Ala Tyr Ala Ile Ser
Thr Leu Ile Lys Gln Lys Leu Asp Gly Leu Lys Asn Glu Gly Leu
Lys Glu Lys Ile Asp Ala Ala Lys Lys Cys Ser Glu Thr Phe Thr
Asn Lys Leu Lys Glu Lys His Thr Asp Leu Gly Lys Glu Gly Val
Thr Asp Ala Asp Ala Lys Glu Ala Ile Leu Lys Thr Asn Gly Thr
Lys Thr Lys Gly Ala Glu Glu Leu Gly Lys Leu Phe Glu Ser Val
Glu Val Leu Ser Lys Ala Ala Lys Glu Met Leu Ala Asn Ser Val
Lys Glu Leu Thr Ser Pro Val Val Ala Glu Ser Pro Lys Lys Pro

Amino-acid sequence of pC protein

Example 8:

Comparison of assay kits with proteins according to the invention and those in which an ultrasonicate was used

20

74 sera from patients with Erythema migrans were
assayed for IgM and IgG antibodies. In addition, a
negative control group of 100 blood donors was tested.
In these assays, on the one hand ultrasonicate
preparations of *Borrelia burgdorferi* were employed in
accordance with methods known per se for carrying out
ELISA assays. On the other hand, recombinant proteins
prepared according to the invention were employed
separately and together. The following tables show
unambiguously that a considerably higher sensitivity
can be achieved by the method according to the inven-
tion than when ultrasonicate is used.

30

DETECTION of IgM antibodies

ELISA/antigen Erythema migrans
(n = 74)

Ultrasonicate	20 27.0%
---------------	----------

p41 (recomb.)	22 29.7%
OspA (recomb.)	7 9.4%
pC (recomb.)	26 35.1%

p41 and/or pC	34 45.9%
---------------	----------

p41 and/or pC and/or OspA	34 45.9%
------------------------------	----------

10

DETECTION of IgG antibodies

ELISA/antigen Erythema migrans
(n = 74)

Ultrasonicate	17 22.9%
---------------	----------

p41 (recomb.)	23 31.1%
OspA (recomb.)	6 8.1%
pC (recomb.)	27 36.5%

p41 and/or pC	34 45.9%
---------------	----------

p41 and/or pC and/or OspA	35 47.3%
------------------------------	----------

20

DETECTION of IgG and/or
IgM antibodies

ELISA/antigen Erythema migrans
(n = 74)

Ultrasonicate	30 40%
p41 (recomb.)	39 53%
OspA (recomb.)	11 15%
pC (recomb.)	41 55%
p41 and/or pC	53 72%
p41 and/or pC and/or OspA	53 72%

10

Description of the tables

Tab. 1:

Reactivity of Lyme borreliosis sera from various stages of the disease with *B. burgdorferi* antigens (p17, pC, p41, p100) in Western blot with *B. burgdorferi* lysate as antigen.

Table 1 summarizes the immunodominant proteins in various stages of Lyme borreliosis.

20

1.1. Sera from healthy people and, to a greater extent, from syphilis patients exhibited antibodies against p60 (common antigen). Antibodies against p41 were found less commonly.

1.2. For early manifestations (EM and LMR), the immunodominant proteins proved to be the flagella protein p41 and the pC protein. pC is the immunodominant protein for the early immune response. In particular, IgM

antibodies against pC may occur earlier than IgM antibodies against p41 (see also Fig. 2a)

1.3. Sera from patients with late manifestations (ACA and arthritis) reacted in all cases (n = 22) with p41 or p100 and in 21 cases with p100 or p17. p17 reacted in 17, p100 in 19 and p41 in 20 cases.

1.4. The intrathecal IgG immune response was directed against p41 in all 12 tested cases. Antibodies against p41 were undetectable in serum in 3 cases.

10

Tab. 2

Reactivity of the immune sera (against various bacterial pathogens) with proteins from *B. burgdorferi* (Western blot).

20

Western blot strips with *B. burgdorferi* lysate fractionated by electrophoresis were prepared as described in Example 1 and incubated with sera against various more or less related and therefore cross-reacting pathogens. The sera were derived from rabbits which had been immunized with the particular pathogens. p100 has the lowest cross-reactivity; only one (anti-*B. hermsii*) of the 15 assayed pathogen-specific sera reacts with this protein. p41 and pC each react with three of the sera and therefore also appear suitable for diagnostic use. The presence of immunoconserved antigens is distinctly evident; thus, for example, 14 and 12, respectively, of the assayed sera react with proteins 40 and 60 kD in size (p40; p60). These common antigens are therefore unsuitable for diagnostic use.

Tab. 1: Immunodominant proteins for the humoral immune response in Lyme borreliosis

1.1 Reactivity of human control sera (IgG Western blot)

	pC	p41	p60	Number
Healthy	-	2	3	17
Syphilis	-	1	5	9

1.2. Immune response to pC and p41 when there is Erythema migrans (EM) and lymphocytic meningoradiculitis (LMR) (Western blot)

10

Diagnosis	Reactive p41	proteins pC	Ig class	Number
EM	11	13	IgM	15 ¹⁾
LMR	13	10	IgM	20 ¹⁾
	14	3	IgG	15 ²⁾

1) The sera were positive in the IgM IFA AB assay.

2) The sera were positive in the IgG IFA AB assay.

1.3. Immune response to p100, p41 and p17 (IgG Western blot)

20

Diagnosis	p100	p41	p17	p100 or p41	p100 or p17	Number
ACA	8	8	9	10	10	10
Arthritis	11	12	8	12	11	12

1.4. Intrathecal immune response when there is lymphocytic meningoradiculitis (IgG Western Blot)

Local intra- Reactivity Number
thecal immune in serum
response

p41	12	9	12
other pro-			
teins	7	12	12

Tab. 2: Reactivity of immune sera (against various bacterial pathogens) with proteins from *B. burgdorferi* (Western blot)

Protein	B. hermsii	T. phagedenis	T. pallidum	L. grippotyphosa	C. jejuni	E. coli	S. typhimurium	Sh. flexneri	Y. enterocolitica O3	Y. enterocolitica O9	P. aeruginosa	H. influenzae	N. meningitidis	L. monocytogenes O1	L. micdadei	Σ
p100	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
p75	+	+	-	+	+	+	+	+	+	+	+	+	-	+	-	12
p70	-	+	+	-	+	-	-	+	+	+	+	+	-	+	+	10
p60	+	+	-	+	+	+	+	+	+	+	+	+	+	-	-	12
p41	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	3
p40	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	14
OspB	+	+	+	-	+	+	-	+	+	-	-	+	-	-	+	9
p33	+	+	+	-	+	+	-	-	+	+	+	+	+	+	-	12
OspA	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
p30	+	+	-	-	-	+	-	-	+	-	-	-	-	+	-	5
p23	+	+	-	-	-	+	+	+	+	+	+	-	-	-	-	8
pC	+	-	-	-	-	-	-	+	-	+	-	-	-	-	-	3
p21	-	+	-	-	-	-	+	+	+	+	+	-	-	-	-	6

Description of the figures

Fig. 1 a and b:

Reactivity of *B. burgdorferi*-infected patients with lysates from 5 different *B. burgdorferi* strains in a Western blot.

10 Sera from stages II and III (neuroborreliosis, stage II (IgM and IgG); acrodermatitis (IgG) and arthritis (IgG), stage III) were assayed. The early immune response is directed, irrespective of the assayed strain, against a narrow spectrum of *Borrelia* proteins (pC and p41). The late immune response is directed against a broad panel of *Borrelia* proteins. Immunodominant proteins are (irrespective of the assayed strain) p100 (with variable molecular weight) and p41.

Fig. 2

2a) Monitoring progress (IgM Western blot) of Erythema migrans

20 The pC protein may be the immunodominant protein of the early immune response. Antibodies against p41 may occur later and be expressed only weakly. IgM antibodies against p17 may also occur when the disease has lasted a long time.

2b) IgG Western blot when there are late manifestations

30 IgG antibodies recognize a broad spectrum of *Borrelia* proteins. The immunodominant proteins when the PKo strain is used prove to be p17 and p100. p17 is strongly expressed by the PKo strain (in contrast to other strains; see Fig. 1). The flagellin p41 was not recognized in 2 of these examples (serum 1 and 2).

Fig. 3

Diagram of DNA amplification of the p41-encoding region A; Section of the *B. burgdorferi* DNA with the p41-encoding region (black bar).

B; Enlargement of the 5' or 3' end of the p41 gene with the relevant DNA sequences. Also indicated is the translation start (ATG) and the stop codon at the 3' end (TAA). The primer sequences used for the PCR are additionally indicated below (primer 1) and above (primer 2) the p41-encoding DNA double-strand. The primers can be hybridized only with the 3' regions in each case. The 5' ends contain non-hybridizing parts which represent cleavage sites for restriction enzymes: GGATCC - BamHI; TCATGA - BspHI, at the 5' end; GACGTC - PstI at the 3' end.

Fig. 4

Expression, reactivity and purification of recombinant p41.

Left side: Coomassie blue-stained SDS polyacrylamide gel. The individual lanes were loaded as follows: 1, E. coli lysate, negative control; 2, E. coli lysate with pUC8ly17 after IPTG induction, the p41 produced by recombination is evident as additional bands in the region of about 45 kDa; 3, supernatant of the lysate from 2 after disruption of the cells as described in Example 4; 4, pellet fraction of the lyzed cells with the recombinant p41; 5, octyl glucopyranoside supernatant; 6, as 5 but pellet fractions; 7-10, fractions after elution of p41 from a MonoQ column by a salt gradient; lanes 9 and 10 contain recombinant p41, owing to degradation events and incomplete translation, besides the complete product there are also smaller fragments which, however, are also to be found in authentic p41 material from *B. burgdorferi*.

Right side: immunostained Western blot of an SDS gel with samples of the Coomassie-stained gel. The immunostaining was carried out with a monoclonal antibody described in Example 6. Labeling of the lanes and of the samples as Coomassie-stained gel; lane 0, empty lane.

Fig. 5

HPLC elution profile of p41 from an ion exchanger column with a salt gradient.

The anion exchanger purification (MonoQ from Pharmacia) of p41 was followed by the antigen being back-dialyzed against 4 M urea without salt and again loaded onto the MonoQ column to check the purity. The elution profile now shows only one protein adsorption peak. The smaller peak immediately in front of the main fraction corresponds to the p41 fragment, with a size of about 30 kD, visible in Fig. 4, lane 8 (assayed by Western blot).

10

Fig. 6:

IgG ELISA with recombinant p41 as antigen.

The recombinant antigen purified on an anion exchanger (MonoQ) (see Fig. 5) was employed in a concentration of 0.5 µg/ml. 7 sera from patients with clinically defined Lyme borreliosis and 8 sera from healthy subjects were assayed. 4 sera from the Lyme borreliosis patients reacted strongly in the Western blot with the recombinant p41 (= positive), 3 sera reacted weakly (= marginal), while sera from the healthy subjects did not react (= negative). The IgG ELISA showed a comparable result. Y axis: optical density at wavelength 486 nm; marg. = marginal

20

Fig. 7

Reactivity of monoclonal antibodies against various *B. burgdorferi* antigens.

Six monoclonal bodies against *B. burgdorferi* were assayed with 30 different *B. burgdorferi* strains, 4 relapsing fever *Borrelia* strains and 2 different *Treponema*. The figure depicts as examples three different *B. burgdorferi* isolates (1 = B31, American strain; 2 = PKo, German skin strain; 3 = PBi, German CSF strain), one relapsing fever *Borrelia* (4 = *B. hermsii*) and one *Treponema* strain (5 = *T. phagedenis*). The monoclonal antibodies prepared as in

30

2072098

Example 6 were employed.

-47-

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1. An immunologically active protein from *Borrelia burgdorferi*, which is in a form free of other proteins originating from *Borellia burgdorferi* and which has been prepared by genetic manipulation, and which has

a) the amino acid sequence

Met Lys Lys Asn Thr Leu Thr Ala Ile Leu Met Thr Leu Phe Leu
Phe Ile Ser Cys Asn Asn Ser Gly Lys Val Gly Ile Leu Thr Ser
Thr Asn Pro Ala Asp Glu Ser Ala Lys Gly Pro Asn Leu Thr Glu
Ile Ser Lys Lys Ile Thr Asp Ser Asn Ala Phe Val Leu Ala Val
Lys Glu Val Glu Thr Leu Val Leu Ser Ile Asp Glu Leu Ala Lys
Lys Ala Ile Gly Gln Lys Ile Asp Asn Asn Asn Gly Leu Ala Ala
Leu Asn Asn Gln Asn Gly Ser Leu Leu Ala Gly Ala Tyr Ala Ile
Ser Thr Leu Ile Thr Glu Lys Leu Ser Lys Leu Lys Asn Leu Glu
Glu Leu Lys Thr Glu Ile Ala Lys Ala Lys Lys Cys Ser Glu Glu
Phe Thr Asn Lys Leu Lys Ser Gly His Ala Asp Leu Gly Lys Gln
Asp Ala Thr Asp Asp His Ala Lys Ala Ala Ile Leu Lys Thr His
Ala Thr Thr Asp Lys Gly Ala Lys Glu Phe Lys Asp Leu Phe Glu
Ser Val Glu Gly Leu Leu Lys Ala Ala Gln Val Ala Leu Thr Asn
Ser Val Lys Glu Leu Thr Ser Pro Val Val Ala Glu Ser Pro Lys
Lys Pro

or an immunologically active protein fragment thereof of
at least 10 amino acids; or

b) the amino acid sequence

Met Lys Lys Asn Thr Leu Ser Ala Ile Leu Met Thr Leu Phe Leu
Phe Ile Ser Cys Asn Asn Ser Gly Lys Asp Gly Asn Thr Ser Ala
Asn Ser Ala Asp Glu Ser Val Lys Gly Pro Asn Leu Thr Glu Ile
Ser Lys Lys Ile Thr Asp Ser Asn Ala Val Leu Leu Ala Val Lys
Glu Val Glu Ala Leu Leu Ser Ser Ile Asp Glu Ile Ala Ala Lys
Ala Ile Gly Lys Lys Ile His Gln Asn Asn Gly Leu Asp Thr Glu
Asn Asn His Asn Gly Ser Leu Leu Ala Gly Ala Tyr Ala Ile Ser
Thr Leu Ile Lys Gln Lys Leu Asp Gly Leu Lys Asn Glu Gly Leu
Lys Glu Lys Ile Asp Ala Ala Lys Lys Cys Ser Glu Thr Phe Thr
Asn Lys Leu Lys Glu Lys His Thr Asp Leu Gly Lys Glu Gly Val
Thr Asp Ala Asp Ala Lys Glu Ala Ile Leu Lys Thr Asn Gly Thr
Lys Thr Lys Gly Ala Glu Glu Leu Gly Lys Leu Phe Glu Ser Val
Glu Val Leu Ser Lys Ala Ala Lys Glu Met Leu Ala Asn Ser Val
Lys Glu Leu Thr Ser Pro Val Val Ala Glu Ser Pro Lys Lys Pro

or an immunologically active protein fragment thereof of
at least 10 amino acids; or

-48-

c) the amino acid sequence

Met Lys Lys Met Leu Leu Ile Phe Ser Phe Phe Leu Val Phe Leu
 Asn Gly Phe Pro Leu Asn Ala Arg Glu Val Asp Lys Glu Lys Leu
 Lys Asp Phe Val Asn Met Asp Leu Glu Phe Val Asn Tyr Lys Gly
 Pro Tyr Asp Ser Thr Asn Thr Tyr Glu Gln Ile Val Gly Ile Gly
 Glu Phe Leu Ala Arg Pro Leu Ile Asn Ser Asn Ser Asn Ser Ser
 Tyr Tyr Gly Lys Tyr Phe Val Asn Arg Phe Ile Asp Asp Gln Asp
 Lys Lys Ala Ser Val Asp Ile Phe Ser Ile Gly Ser Lys Ser Glu
 Leu Asp Ser Ile Leu Asn Leu Arg Arg Ile Leu Thr Gly Tyr Leu
 Ile Lys Ser Phe Asp Tyr Glu Arg Ser Ser Ala Glu Leu Ile Ala
 Lys Ala Ile Thr Ile Tyr Asn Ala Val Tyr Arg Gly Asp Leu Asp
 Tyr Tyr Lys Glu Phe Tyr Ile Glu Ala Ser Leu Lys Ser Leu Thr
 Lys Glu Asn Ala Gly Leu Ser Arg Val Tyr Ser Gln Trp Ala Gly
 Lys Thr Gln Ile Phe Ile Pro Leu Lys Lys Asn Ile Leu Ser Gly
 Asn Val Glu Ser Asp Ile Asp Ile Asp Ser Leu Val Thr Asp Lys
 Val Val Ala Ala Leu Leu Ser Glu Asn Glu Ser Gly Val Asn Phe
 Ala Arg Asp Ile Thr Asp Ile Gln Gly Glu Thr His Lys Ala Asp
 Gln Asp Lys Ile Asp Ile Glu Leu Asp Asn Phe His Glu Ser Asp
 Ser Asn Ile Thr Glu Thr Ile Glu Asn Leu Arg Asp Gln Leu Glu
 Lys Ala Thr Asp Glu Glu His Lys Lys Glu Ile Glu Ser Gln Val
 Asp Ala Lys Lys Lys Gln Lys Glu Glu Leu Asp Lys Lys Ala Ile
 Asp Leu Asp Lys Ala Gln Gln Lys Leu Asp Phe Ala Glu Asp Asn
 Leu Asp Ile Gln Arg Asp Thr Val Arg Glu Lys Leu Gln Glu Asn
 Ile Asn Glu Thr Asn Lys Glu Lys Asn Leu Pro Lys Pro Gly Asp
 Val Ser Ser Pro Lys Val Asp Lys Gln Leu Gln Ile Lys Glu Ser
 Leu Glu Asp Leu Gln Glu Gln Leu Lys Glu Ala Ser Asp Glu Asn
 Gln Lys Arg Glu Ile Glu Lys Gln Ile Glu Ile Lys Lys Asn Asp
 Glu Glu Leu Phe Lys Asn Lys Asp His Lys Ala Leu Asp Leu Lys
 Gln Glu Leu Asn Ser Lys Ala Ser Ser Lys Glu Lys Ile Glu Gly
 Glu Glu Glu Asp Lys Glu Leu Asp Ser Lys Lys Asn Leu Glu Pro
 Val Ser Glu Ala Asp Lys Val Asp Lys Ile Ser Lys Ser Asn Asn
 Asn Glu Val Ser Lys Leu Ser Pro Leu Asp Glu Pro Ser Tyr Ser
 Asp Ile Asp Ser Lys Glu Gly Val Asp Asn Lys Asp Val Asp Leu
 Gln Lys Thr Lys Pro Gln Val Glu Ser Gln Pro Thr Ser Leu Asn
 Glu Asp Leu Ile Asp Val Ser Ile Asp Ser Ser Asn Pro Val Phe
 Leu Glu Val Ile Asp Pro Ile Thr Asn Leu Gly Thr Leu Gln Leu
 Ile Asp Leu Asn Thr Gly Val Arg Leu Lys Glu Ser Ala Gln Gln
 Gly Ile Gln Arg Tyr Gly Ile Tyr Glu Arg Glu Lys Asp Leu Val
 Val Ile Lys Ile Asp Ser Gly Lys Ala Lys Leu Gln Ile Leu Asp
 Lys Leu Glu Asn Leu Lys Val Ile Ser Glu Ser Asn Phe Glu Ile
 Asn Lys Asn Ser Ser Leu Tyr Val Asp Ser Arg Met Ile Leu Val
 Val Val Lys Asp Asp Ser Asn Ala Trp Arg Leu Ala Lys Phe Ser
 Pro Lys Asn Leu Asp Glu Phe Ile Leu Ser Glu Asn Lys Ile Leu
 Pro Phe Thr Ser Phe Ala Val Arg Lys Asn Phe Ile Tyr Leu Gln
 Asp Glu Leu Lys Ser Leu Val Thr Leu Asp Val Asn Thr Leu Lys
 Lys Val Lys

or an immunologically active protein fragment thereof of at least 10 amino acids.

-49-

2. An immunologically active protein as claimed in claim 1, which can be prepared using DNA isolated from *Borrelia burgdorferi*.
3. An immunologically active protein as claimed in claim 2, which can be prepared using DNA isolated from *Borrelia burgdorferi* (DSM No. 5662).
4. An assay kit for detecting antibodies against *Borrelia* strains, which contains at least one immunologically active protein as claimed in any of claims 1 to 3, which protein is able to react with the antibodies present in a test fluid, and which kit has at least one indicator component which makes it possible to detect complexes of immunologically active protein and antibody.
5. An assay kit as claimed in claim 4, which contains 2 to 4 immunologically active proteins as claimed in claims 1 to 3.
6. An assay kit as claimed in either of claims 4 or 5, wherein the indicator component is an antibody which is directed against the antibody to be detected and which has a label.
7. An assay kit as claimed in claim 6, wherein the label comprises a radioactive isotope.
8. An assay kit as claimed in claim 6, wherein the label comprises an enzyme which is able to catalyze a colour reaction.
9. An assay kit as claimed in claims 4 or 5, wherein the immunologically active protein is biotinylated, and the

-50-

indicator component is avidin or streptavidin with an enzyme covalently bonded thereto.

10. An assay kit as claimed in claims 4 or 5, including a monoclonal antibody directed against the immunologically active protein, said monoclonal antibody being biotinylated, and the indicator component is avidin or streptavidin with an enzyme covalently bonded thereto.
11. An assay kit as claimed in claims 9 or 10, wherein the indicator component is avidin or streptavidin with peroxidase covalently bonded thereto.
12. An assay kit as claimed in any of claims 4 to 6, which is an ELISA assay kit.
13. An assay kit as claimed in claim 4 or 5, wherein at least one immunologically active protein as claimed in any of claims 1 to 3 is coupled to microtiter plates, and the indicator component comprises anti-human immunoglobulin, to which an enzyme catalyzing a colour reaction is coupled.
14. An assay kit as claimed in claim 13, wherein the indicator component comprises one or both of IgG or IgM antibodies, to which an enzyme catalyzing a colour reaction is coupled.
15. The use of immunologically active proteins as claimed in any of claims 1 to 3 for preparing vaccines for protection against infections by *Borrelia* bacteria.
16. The use of immunologically active proteins as claimed in claim 15, for preparing vaccines for protection against infections by *Borrelia burgdorferi* bacteria.

Fig. 1: IgG Western blot with 5 different strains as antigen

IgG and IgM response in stage II

IgG response in stage III

Neuroborreliosis, stage II
(IgM) (IgG)

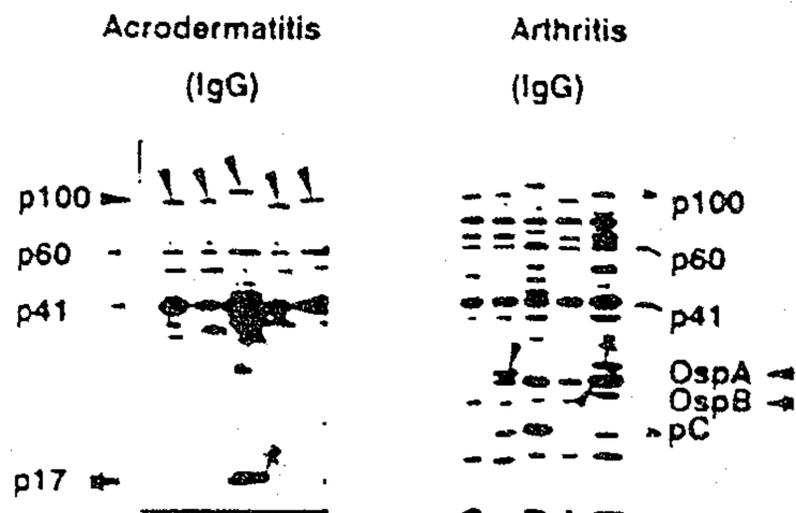
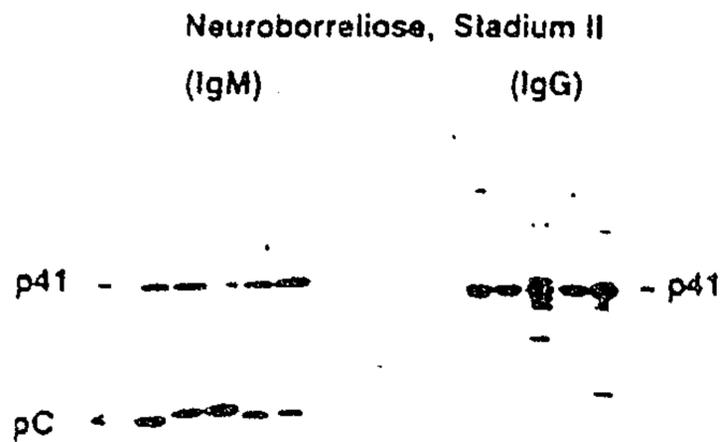
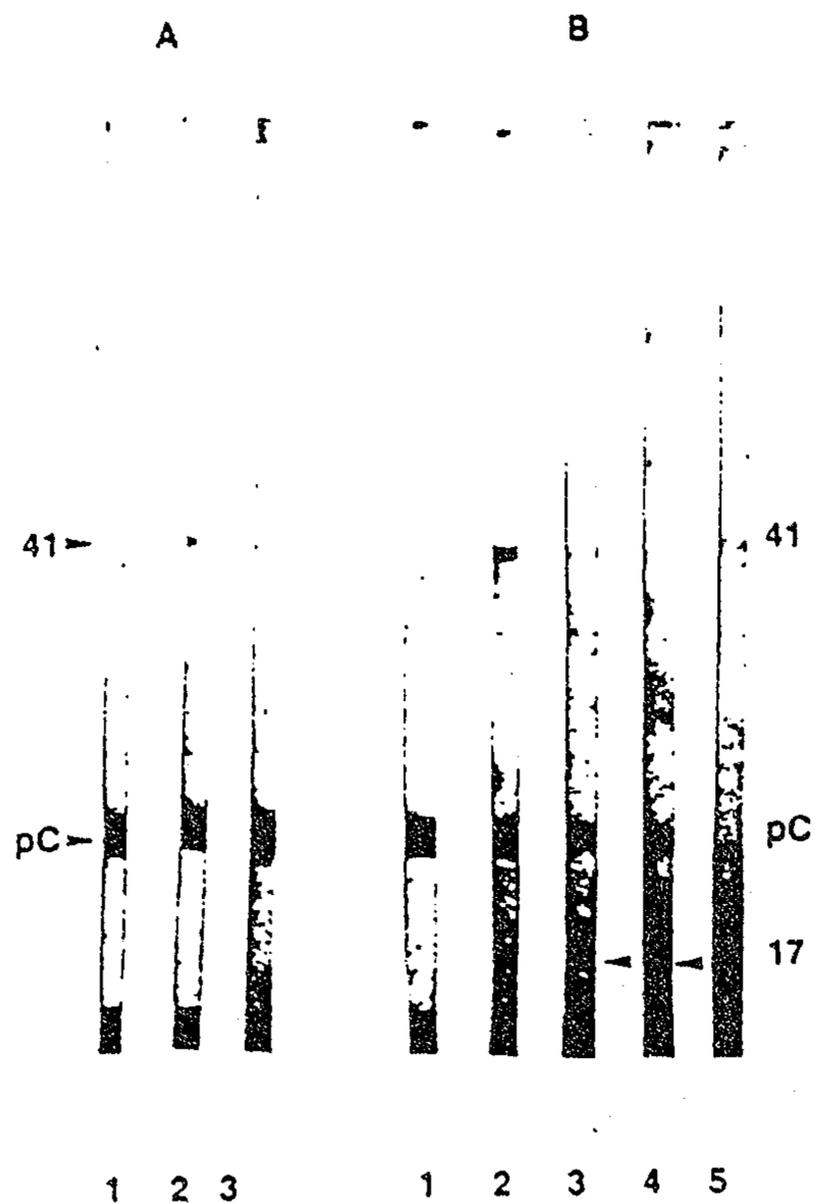


Fig. 2a: IgM Western blot when there is Erythema migrans

Antigen: *B. burgdorferi*/PKo strain

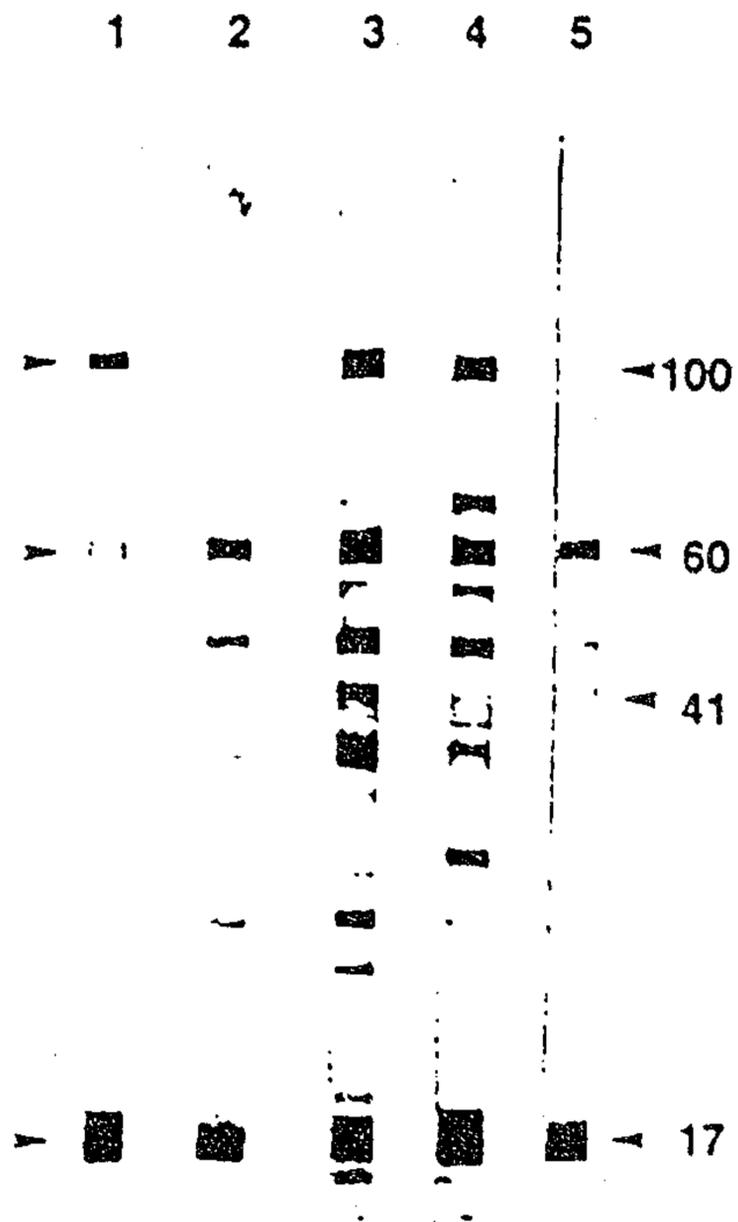


Patient A: 1 First test
 2,3 Monitoring progress after 1 and 3 weeks

Patient B: 1 First test
 2-5 Monitoring progress after 2, 9, 10 and 11 weeks

Fig. 2b: IgG Western blot when there are late manifestations

Antigen: *B. burgdorferi*/PKo strain



1, 2, 3

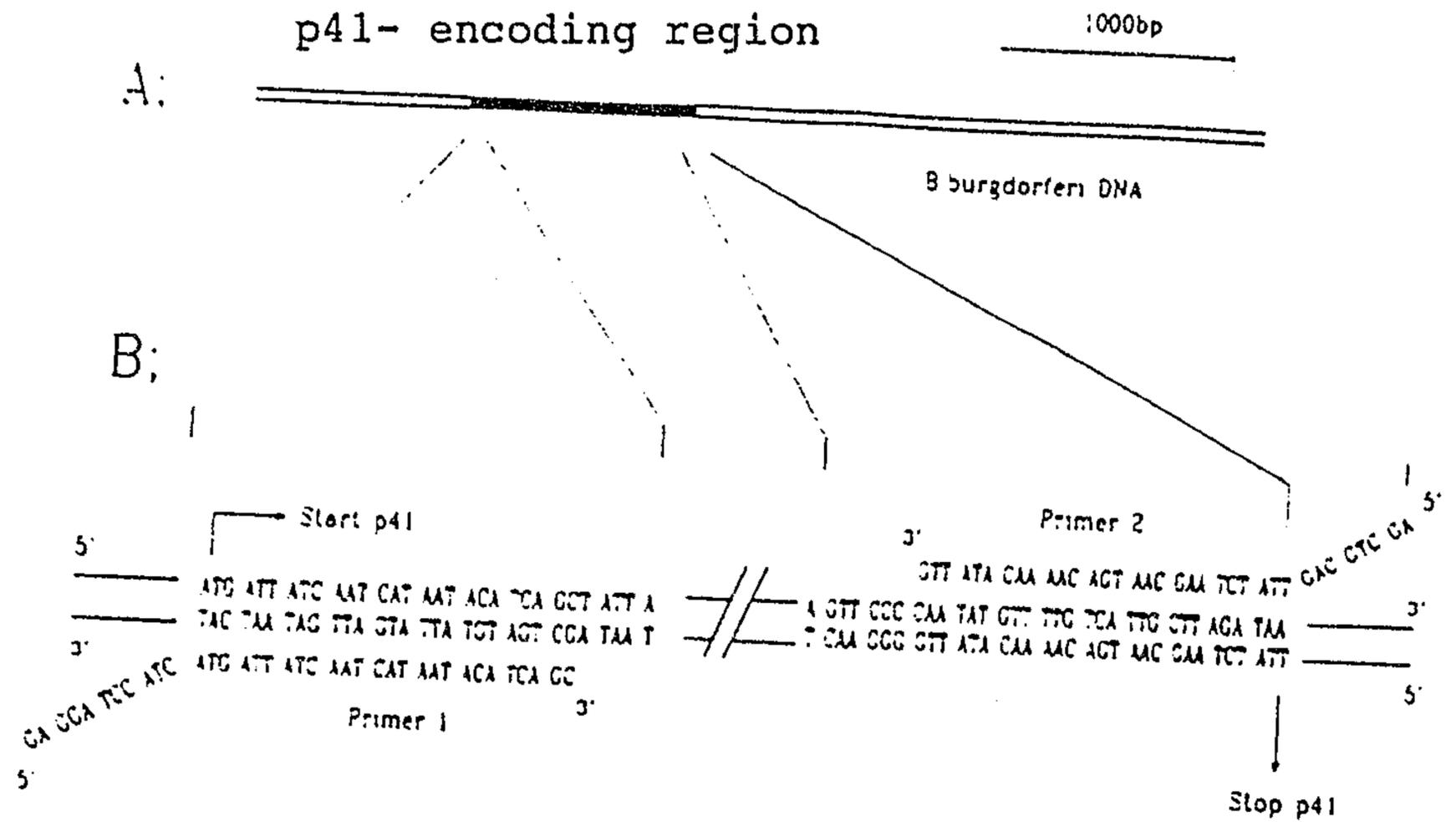
Acrodermatitis

4, 5

Arthritis

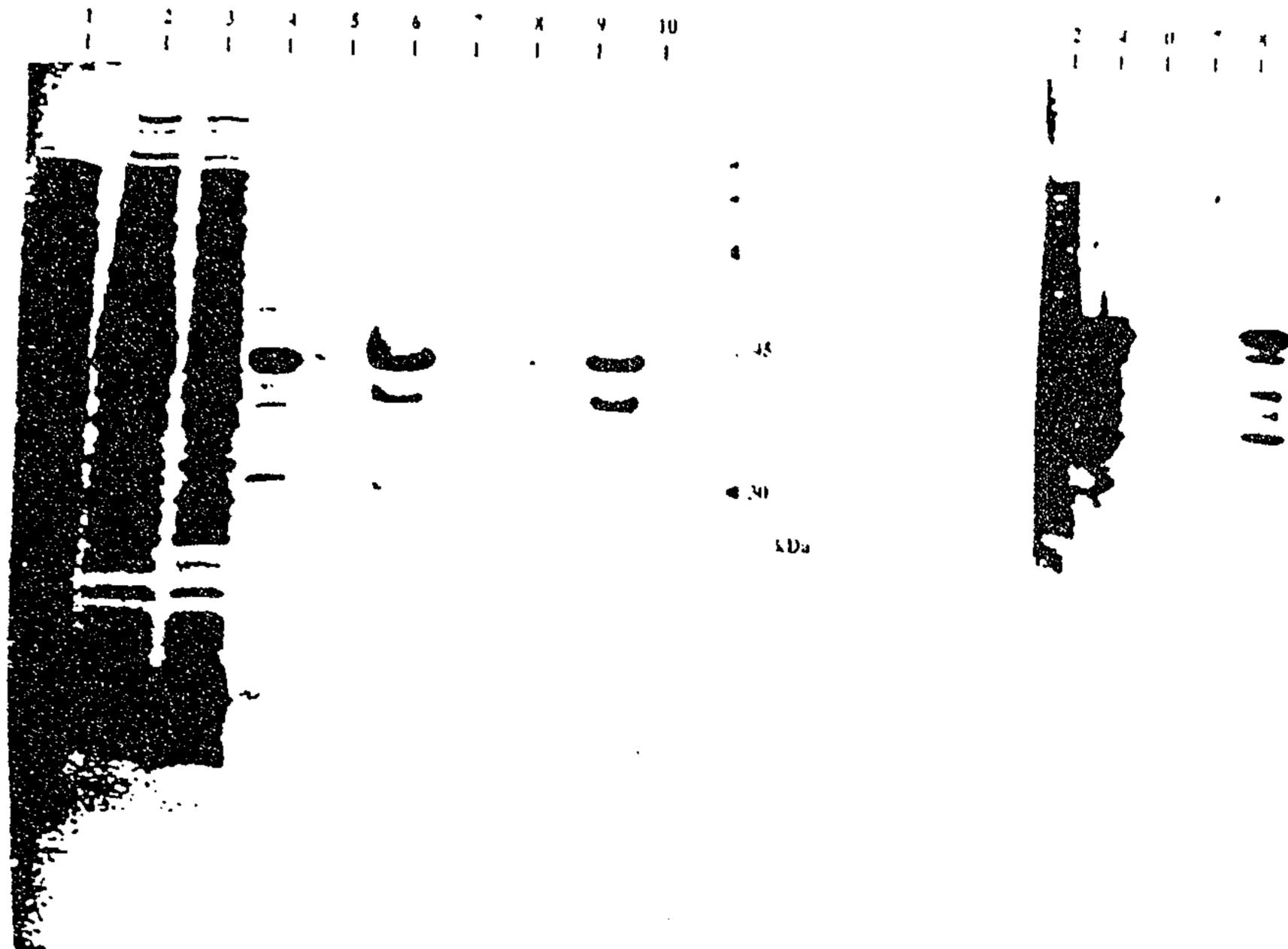
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Fig. 3



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Fig. 4



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Fig. 5

Elution profile of p41 from an ion exchanger column

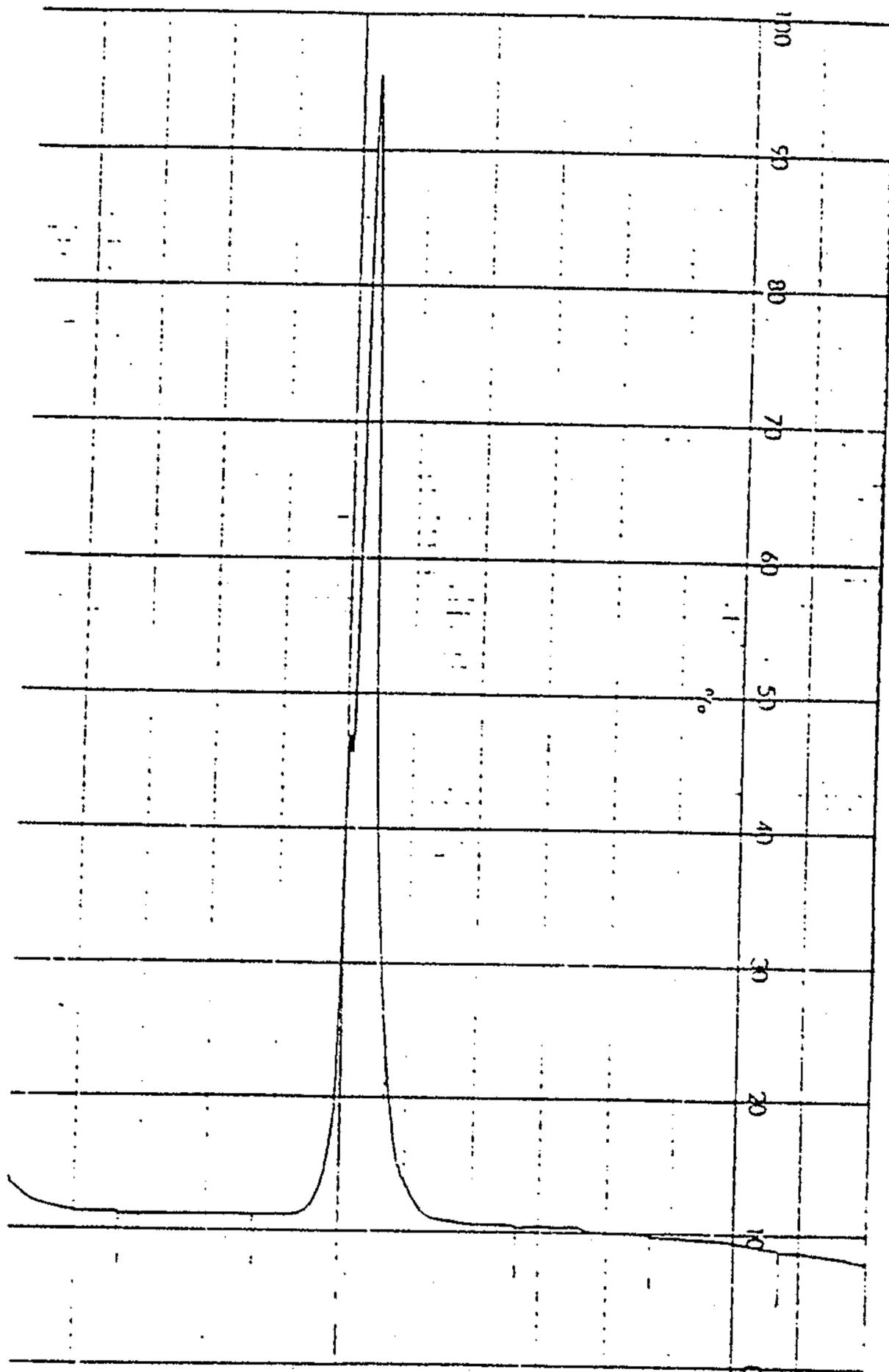


Fig. 6

IgG - ELISA with recombinant p41 as antigen (0.5 μ g/ml)

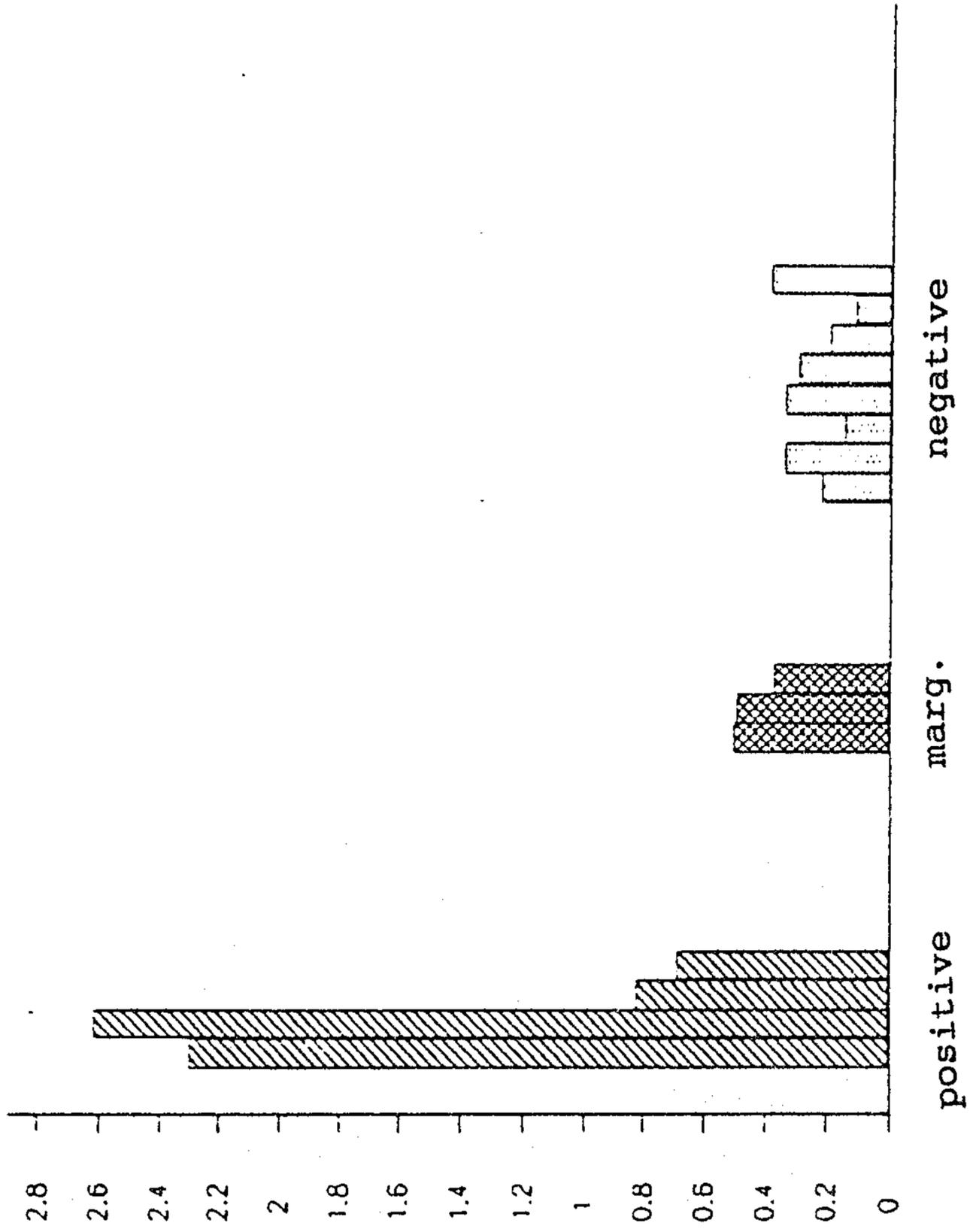


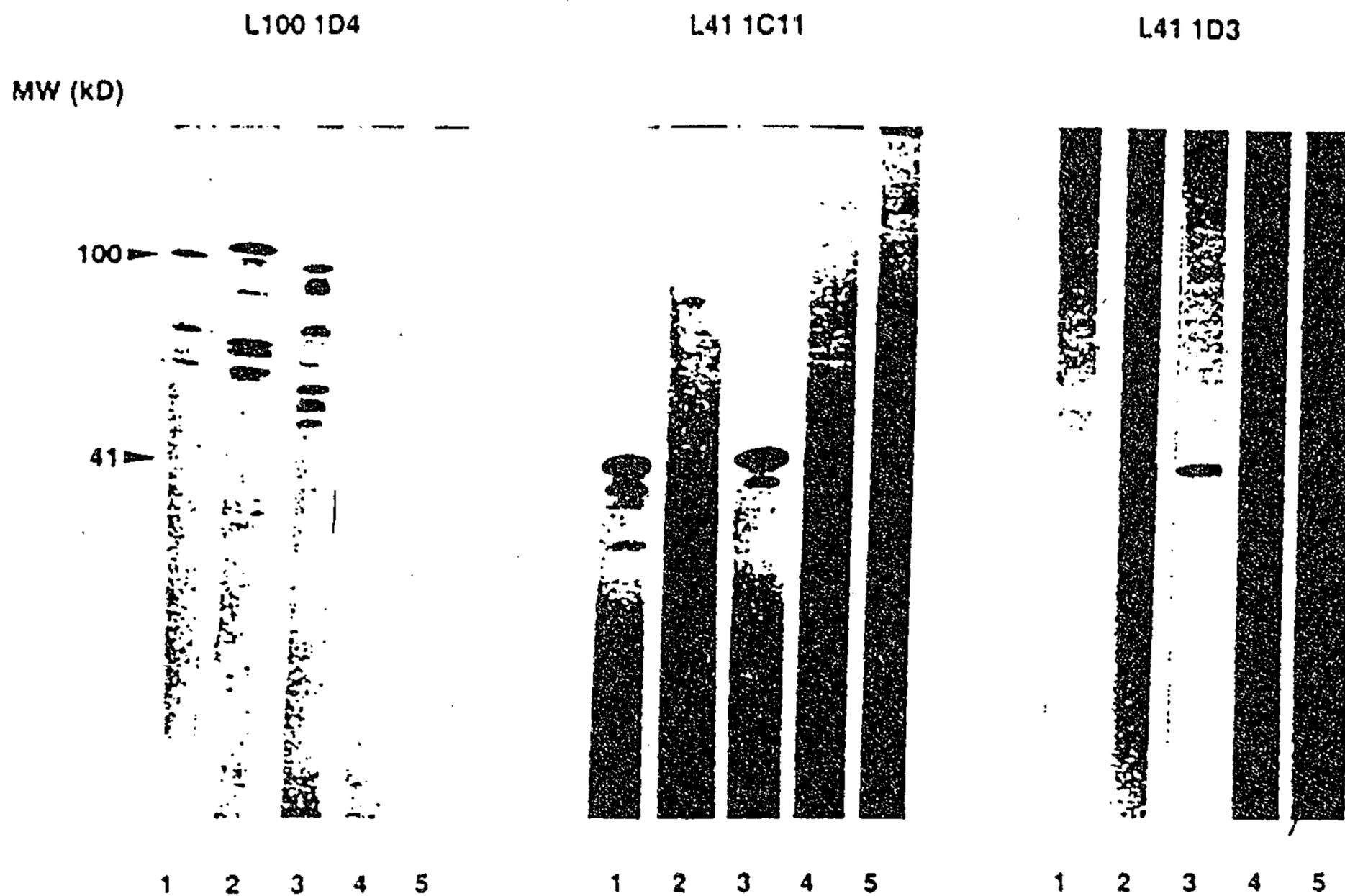
Fig. 7a: Monoclonal antibodies against *B. burgdorferi*

Fig. 7b: Monoclonal antibodies against *B. burgdorferi*