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(54) **VACCINS CONTRE LA MALADIE DE LYME**
(54) **LYME DISEASE VACCINES**

(57) Cette invention se rapporte à de nouveaux vaccins permettant de prévenir ou d'atténuer la maladie de Lyme. Cette invention se rapporte en outre à des molécules d'acides nucléiques isolées codant des polypeptides antigéniques de Borrelia burgdorferi. Cette invention présente également des polypeptides antigéniques, ainsi que des vecteurs, de cellules hôtes et des procédés de recombinaison pour la production de ceux-ci. Cette invention se rapporte en outre à des procédés de diagnostic pour détecter l'expression de gènes de Borrelia.

(57) The present invention relates to novel vaccines for the prevention or attenuation of Lyme disease. The invention further relates to isolated nucleic acid molecules encoding antigenic polypeptides of Borrelia burgdorferi. Antigenic polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The invention additionally relates to diagnostic methods for detecting Borrelia gene expression.

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(57) Abstract

The present invention relates to novel vaccines for the prevention or attenuation of Lyme disease. The invention further relates to isolated nucleic acid molecules encoding antigenic polypeptides of *Borrelia burgdorferi*. Antigenic polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The invention additionally relates to diagnostic methods for detecting *Borrelia* gene expression.

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Lyme Disease Vaccines

Field of the Invention

The present invention relates to novel vaccines for the prevention or attenuation of Lyme disease. The invention further relates to isolated nucleic acid molecules encoding antigenic polypeptides of *Borrelia burgdorferi*. Antigenic polypeptides are also provided, as are vectors, host cells and recombinant methods for producing the same. The invention additionally relates to diagnostic methods for detecting *Borrelia* gene expression.

Background of the Invention

Lyme disease (Steere, A.C., *Proc. Natl. Acad. Sci. USA 91*:2378-2383 (1991)), or Lyme borreliosis, is presently the most common human disease in the United States transmitted by an arthropod vector (Center for Disease Control, *Morbid. Mortal. Weekly Rep. 46(23)*:531-535 (1997)). Further, infection of house-hold pets, such as dogs, is a considerable problem.

While initial symptoms often include a rash at the infection point, Lyme disease is a multisystemic disorder that may include arthritic, carditic, and neurological manifestations. While antibiotics are currently used to treat active cases of Lyme disease, *B. burgdorferi* persists even after prolonged antibiotic treatment. Further, *B. burgdorferi* can persist for years in a mammalian host in the presence of an active immune response (Straubinger, R. *et al., J. Clin. Microbiol. 35*:111-116 (1997); Steere, A., *N. Engl. J. Med. 321*:586-596 (1989)).

Lyme disease is caused by the related tick-borne spirochetes classified as *Borrelia burgdorferi* sensu lato (including *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*). Although substantial progress has been made in the biochemical, ultrastructural, and genetic characterization of the organism, the spirochetal factors responsible for infectivity, immune evasion and disease pathogenesis remain largely obscure.

A number of antigenic *B. burgdorferi* cell surface proteins have been identified. These include the outer membrane surface proteins (Osp) OspA, OspB, OspC and OspD. OspA and OspB are encoded by tightly linked tandem genes which are transcribed as a single transcriptional unit (Brusca, J. *et al., J. Bacteriol. 173*:8004-8008 (1991)). The most-studied *B. burgdorferi* membrane protein is OspA, a lipoprotein antigen expressed by borreliae in resting ticks and the most abundant protein expressed *in vitro* by most borrelial isolates (Barbour, A.G., *et al., Infection & Immunity 41*:795-804 (1983); Howe, T.R., *et al., Science 227*:645 (1985)).

A number of different types of Lyme disease vaccines have been shown to induce immunological responses. Whole-cell *B. burgdorferi* vaccines, for example, have been shown to induce both immunological responses and protective immunity in several animal models (Reviewed in Wormser, G., *Clin. Infect. Dis. 21*:1267-1274 (1995)). Further, passive immunity has been demonstrated in both humans and other animals using *B. burgdorferi* specific antisera.

While whole-cell Lyme disease vaccines confer protective immunity in animal models, use of such vaccines presents the risk that responsive antibodies will produce an autoimmune response (Reviewed in Wormser, G., *supra*). This problem is at least partly the result of the production of *B. burgdorferi* specific antibodies which cross-react with hepatocytes and both muscle and nerve cells. *B. burgdorferi* heat shock proteins and the 41-kd flagellin subunit are believed to contain antigens which elicit production of these cross-reactive antibodies.

Single protein subunit vaccines for Lyme disease have also been tested. The cell surface proteins of *B. burgdorferi* are potential candidates for use in such vaccines and several have been shown to elicit protective immune responses in mammals (Probert, W. *et al., Vaccine 15*:15-19 (1997); Fikrig, E. *et al., Infect. Immun. 63*:1658-1662 (1995); Langerman S. *et al., Nature 372*:552-556 (1994); Fikrig, E. *et al., J. Immunol. 148*:2256-2260 (1992)). Experimental OspA vaccines, for example, have demonstrated efficacy in several animal models (Fikrig, E., *et al., Proc. Natl. Acad. Sci. USA 89*:5418-5421 (1992); Johnson, B.J., *et al., Vaccine 13*:1086-1094 (1996); Fikrig, E., *et al., Infect. Immun. 60*:657-661 (1992); Chang, Y.F., *et al., Infection & Immunity 63*:3543-3549 (1995)), and OspA vaccines for human use are under clinical evaluation (Keller, D., *et al., J. Am. Med. Assoc. 271*:1764-1768 (1994); Van Hoecke, C., *et al., Vaccine 14*:1620-1626 (1996)). Passive immunity is also conferred by antisera containing antibodies specific for the full-length OspA protein. Further, vaccination with plasmid DNA encoding OspA has been demonstrated to elicit protective immune responses in mice (Luke, C. *et al., J. Infect. Dis. 175*:91-97 (1997); Zhong, W. *et al., Eur. J. Immunol. 26*:2749-2757 (1996)).

Recent immunofluorescence assay observations indicate that during tick engorgement the expression of OspA by borreliae diminishes (deSilva, A.M., *et al., J. Exp. Med. 183*:271-275 (1996)) while expression of other proteins, exemplified by OspC, increases (Schwan, T.G., *et al., Proc. Natl. Acad. Sci. USA 92*:2909-2913 (1985)). By the time of transmission to hosts, spirochetes in the tick salivary glands express little or no OspA. This down-modulation of OspA appears to explain the difficulties in demonstrating immune responses to this antigen early in infection following tick bites (Kalish, R.A., *et al., Infect. Immun. 63*:2228-2235 (1995); Gern, L., *et al., J. Infect. Dis. 167*:971-975 (1993); Schiable, U.E., *et al., Immunol. Lett. 36*:219-226 (1993)) or following challenge with limiting doses of cultured borreliae (Schiable, U.E., *et al., Immunol. Lett. 36*:219-226 (1993); Barthold, S.W. and Bockenstedt, L.K., *Infect. Immun. 61*:4696-4702 (1993)).

Furthermore, OspA-specific antibodies are ineffective if administered after a borrelial

OspA vaccines must elicit protective levels of antibody which are maintained throughout periods of tick exposure in order to block borrelia transmission from the arthropod vector.

Vaccines in current use against other pathogens include *in vivo*-expressed antigens which could boost anamnestic responses upon infection, potentiate the action of immune effector cells and complement, and inhibit key virulence mechanisms. OspC is both expressed during infection (Montgomery, R.R., *et al., J. Exp. Med. 183*:261-269 (1996)) and a target for protective immunity (Gilmore, R.D., *et al., Infect. Immun. 64*:2234-2239 (1996); Probert, W.S. and LeFebvre, R.B., *Infect. Immun. 62*:1920-1926 (1994); Preac-Mursic, V., *et al., Infection 20*:342-349 (1992)), but mice immunized with this protein were only protected against challenge with the homologous borrelial isolate (Probert, W.S., *et al., J. Infect. Dis. 175*:400-405 (1997)). Identification of *in vivo*-expressed, and broadly protective, antigens of *B. burgdorferi* has remained elusive.

Summary of the Invention

The present invention provides isolated nucleic acid molecules comprising polynucleotides encoding the *B. burgdorferi* peptides having the amino acid sequences shown in Table 1. Thus, one aspect of the invention provides isolated nucleic acid molecules comprising polynucleotides having a nucleotide sequence selected from the group consisting of: (a) a nucleotide sequence encoding any of the amino acid sequences of the full-length polypeptides shown in Table 1; (b) a nucleotide sequence encoding any of the amino acid sequences of the full-length polypeptides shown in Table 1 but minus the N-terminal methionine residue, if present; (c) a nucleotide sequence encoding any of the amino acid sequences of the truncated polypeptides shown in Table 1; and (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c) above.

Further embodiments of the invention include isolated nucleic acid molecules that comprise a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical, to any of the nucleotide sequences in (a), (b), (c), or (d) above, or a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide in (a), (b), (c), or (d) above. This polynucleotide which hybridizes does not hybridize under stringent hybridization conditions to a polynucleotide having a nucleotide sequence consisting of only A residues or of only T residues. Additional nucleic acid embodiments of the invention relate to isolated nucleic acid molecules comprising polynucleotides which encode the amino acid sequences of epitope-bearing portions of a *B. burgdorferi* polypeptide having an amino acid sequence in (a), (b), or (c) above.

The present invention also relates to recombinant vectors, which include the isolated nucleic acid molecules of the present invention, and to host cells containing the recombinant vectors, as well as to methods of making such vectors and host cells and for using these vectors for the production of *B. burgdorferi* polypeptides or peptides by recombinant techniques.

The invention further provides isolated *B. burgdorferi* polypeptides having amino acid

sequence selected from the group consisting of: (a) an amino acid sequence of any of the full-length polypeptides shown in Table 1; (b) an amino acid sequence of any of the full-length polypeptides shown in Table 1 but minus the N-terminal methionine residue, if present; (c) an amino acid sequence of any of the truncated polypeptides shown in Table 1; and (d) an amino acid sequence of an epitope-bearing portion of any one of the polypeptides of (a), (b), or (c).

The polypeptides of the present invention also include polypeptides having an amino acid sequence with at least 70% similarity, and more preferably at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% similarity to those described in (a), (b), (c), or (d) above, as well as polypeptides having an amino acid sequence at least 70% identical, more preferably at least 75% identical, and still more preferably 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% identical to those above; as well as isolated nucleic acid molecules encoding such polypeptides.

The present invention further provides a vaccine, preferably a multi-component vaccine comprising one or more of the *B. burgdorferi* polypeptides shown in Table 1, or fragments thereof, together with a pharmaceutically acceptable diluent, carrier, or excipient, wherein the *B. burgdorferi* polypeptide(s) are present in an amount effective to elicit an immune response to members of the *Borrelia* genus in an animal. The *B. burgdorferi* polypeptides of the present invention may further be combined with one or more immunogens of one or more other borrelial or non-borrelial organisms to produce a multi-component vaccine intended to elicit an immunological response against members of the *Borrelia* genus and, optionally, one or more non-borrelial organisms.

The vaccines of the present invention can be administered in a DNA form, *e.g.*, "naked" DNA, wherein the DNA encodes one or more borrelial polypeptides and, optionally, one or more polypeptides of a non-borrelial organism. The DNA encoding one or more polypeptides may be constructed such that these polypeptides are expressed fusion proteins.

The vaccines of the present invention may also be administered as a component of a genetically engineered organism. Thus, a genetically engineered organism which expresses one or more *B. burgdorferi* polypeptides may be administered to an animal. For example, such a genetically engineered organism may contain one or more *B. burgdorferi* polypeptides of the present invention intracellularly, on its cell surface, or in its periplasmic space. Further, such a genetically engineered organism may secrete one or more *B. burgdorferi* polypeptides.

The vaccines of the present invention may be co-administered to an animal with an immune system modulator (*e.g.*, CD86 and GM-CSF).

The invention also provides a method of inducing an immunological response in an animal to one or more members of the *Borrelia* genus, *e.g.*, *B. burgdorferi* sensu stricto, *B. afzelii,* and *B. garinii*, comprising administering to the animal a vaccine as described above.

The invention further provides a method of inducing a protective immune response in an animal, sufficient to prevent or attenuate an infection by members of the *Borrelia* genus,

be conjugated to another immunogen and/or administered in admixture with an adjuvant.

The invention further relates to antibodies elicited in an animal by the administration of one or more *B. burgdorferi* polypeptides of the present invention.

The invention also provides diagnostic methods for detecting the expression of genes of members of the *Borrelia* genus in an animal. One such method involves assaying for the expression of a gene encoding *Borrelia* peptides in a sample from an animal. This expression may be assayed either directly (*e.g.*, by assaying polypeptide levels using antibodies elicited in response to amino acid sequences shown in Table 1) or indirectly (*e.g.*, by assaying for antibodies having specificity for amino acid sequences shown in Table 1). An example of such a method involves the use of the polymerase chain reaction (PCR) to amplify and detect *Borrelia* nucleic acid sequences.

The present invention also relates to nucleic acid probes having all or part of a nucleotide sequence shown in Table 1 which are capable of hybridizing under stringent conditions to *Borrelia* nucleic acids. The invention further relates to a method of detecting one or more *Borrelia* nucleic acids in a biological sample obtained from an animal, said one or more nucleic acids encoding *Borrelia* polypeptides, comprising:

a) contacting the sample with one or more of the above-described nucleic acid probes, under conditions such that hybridization occurs, and

b) detecting hybridization of said one or more probes to the *Borrelia* nucleic acid present in the biological sample.

Detailed Description

The present invention relates to recombinant antigenic *B. burgdorferi* polypeptides and fragments thereof. The invention also relates to methods for using these polypeptides to produce immunological responses and to confer immunological protection to disease caused by members of the genus *Borrelia*. The invention further relates to nucleic acid sequences which encode antigenic *B. burgdorferi* polypeptides and to methods for detecting *Borrelia* nucleic acids and polypeptides in biological samples. The invention also relates to *Borrelia* specific antibodies and methods for detecting such antibodies produced in a host animal.

Definitions

The following definitions are provided to clarify the subject matter which the inventors consider to be the present invention.

As used herein, the phrase "pathogenic agent" means an agent which causes a disease state or affliction in an animal. Included within this definition, for examples, are bacteria, protozoans, fungi, viruses and metazoan parasites which either produce a disease state or render an animal infected with such an organism susceptible to a disease state (*e.g.*, a secondary infection). Further included are species and strains of the genus *Borrelia* which produce disease states in animals.

As used herein, the term "organism" means any living biological system, including viruses, regardless of whether it is a pathogenic agent.

As used herein, the term "*Borrelia*" means any species or strain of bacteria which is members of the genus *Borrelia*. Included within this definition are *Borrelia burgdorferi* sensu lato (including *B. burgdorferi* sensu stricto, *B. afzelii*, *B. garinii*), *B. andersonii*, *B. anserina*, *B. japonica*, *B. coriaceae*, and other members of the genus *Borrelia* regardless of whether they are known pathogenic agents.

As used herein, the phrase "one or more *B. burgdorferi* polypeptides of the present invention" means the amino acid sequence of one or more of the *B. burgdorferi* polypeptides disclosed in Table 1. These polypeptides may be expressed as fusion proteins wherein the *B. burgdorferi* polypeptides of the present invention are linked to additional amino acid sequences which may be of borrelial or non-borrelial origin. This phrase further includes fragments of the *B. burgdorferi* polypeptides of the present invention.

As used herein, the phrase "full-length amino acid sequence" and "full-length polypeptide" refer to an amino acid sequence or polypeptide encoded by a full-length open reading frame (ORF). An ORF may be defined as a nucleotide sequence bounded by stop codons which encodes a putative polypeptide. An ORF may also be defined as a nucleotide sequence within a stop codon bounded sequence which contains an initiation codon (*e.g.*, a methionine or valine codon) on the 5' end and a stop codon on the 3' end.

As used herein, the phrase "truncated amino acid sequence" and "truncated polypeptide" refer to a sub-sequence of a full-length amino acid sequence or polypeptide. Several criteria may also be used to define the truncated amino acid sequence or polypeptide. For example, a truncated polypeptide may be defined as a mature polypeptide (*e.g.*, a polypeptide which lacks a leader sequence). A truncated polypeptide may also be defined as an amino acid sequence which is a portion of a longer sequence that has been selected for ease of expression in a heterologous system but retains regions which render the polypeptide useful for use in vaccines (*e.g.*, antigenic regions which are expected to elicit a protective immune response).

Additional definitions are provided throughout the specification.

Explanation of Table 1

Table 1 lists *B. burgdorferi* nucleotide and amino acid sequences of the present invention. The nomenclature used therein is as follows:

"nt" refers to nucleotide sequences;

"aa" refers to amino acid sequences;

"f" refers to full-length nucleotide or amino acid sequences; and

"t" refers to truncated nucleotide or amino acid sequences.

Thus, for example, the designation "f101.aa" refers to the full-length amino acid sequence of *B. burgdorferi* polypeptide number 101. Further, "f101.nt" refers to the full-length nucleotide sequence encoding the full-length amino acid sequence of *B. burgdorferi* polypeptide number 101.

Explanation of Table 2

Table 2 lists accession numbers for the closest matching sequences between the polypeptides of the present invention and those available through GenBank and GeneSeq databases. These reference numbers are the database entry numbers commonly used by those of skill in the art, who will be familar with their denominations. The descriptions of the numenclature for GenBank are available from the National Center for Biotechnology Information. Column 1 lists the gene or ORF of the present invention. Column 2 lists the accession number of a "match" gene sequence in GenBank or GeneSeq databases. Column 3 lists the description of the "match" gene sequence. Columns 4 and 5 are the high score and smallest sum probability, respectively, calculated by BLAST. Polypeptides of the present invention that do not share significant identity/similarity with any polypeptide sequences of GenBank and GeneSeq are not represented in Table 2. Polypeptides of the present invention that share significant identity/similarity with more than one of the polypeptides of GenBank and GeneSeq are represented more than once.

Explanation of Table 3.

The *B. burgdorferi* polypeptides of the present invention may include one or more conservative amino acid substitutions from natural mutations or human manipulation as indicated in Table 3. Changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein. Residues from the following groups, as indicated in Table 3, may be substituted for one another: Aromatic, Hydrophobic, Polar, Basic, Acidic, and Small,

Explanation of Table 4

Table 4 lists residues comprising antigenic epitopes of antigenic epitope-bearing fragments present in each of the full length *B. burgdorferi* polypeptides described in Table 1 as predicted by the inventors using the algorithm of Jameson and Wolf, (1988) Comp. Appl. Biosci. 4:181-186. The Jameson-Wolf antigenic analysis was performed using the computer program PROTEAN (Version 3.11 for the Power MacIntosh, DNASTAR, Inc., 1228 South Park Street Madison, WI). *B. burgdorferi* polypeptide shown in Table 1 may one or more antigenic epitopes comprising residues described in Table 4. It will be appreciated that depending on the analytical criteria used to predict antigenic determinants, the exact address of the determinant may vary slightly. The residues and locations shown described in Table 4 correspond to the amino acid sequences for each full length gene sequence shown in Table 1 and in the Sequence Listing. Polypeptides of the present invention that do not have antigenic epitopes recognized by the Jameson-Wolf algorithm are not represented in Table 2.

Selection of Nucleic Acid Sequences Encoding Antigenic B. burgdorferi Polypeptides

The present invention provides a select number of ORFs from those presented in the fragments of the *Borrelia burgdorferi* genome which may prove useful for the generation of a protective immune response. The sequenced *B. burgdorferi* genomic DNA was obtained from a sub-cultured isolate of ATCC Deposit No. 35210. The sub-cultured isolate was deposited on August 8, 1997 at the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, and given accession number 202012.

Some ORFs contained in the subset of fragments of the *B. burgdorferi* genome disclosed herein were derived through the use of a number of screening criteria detailed below. The ORFs are generally bounded at the amino terminus by a methionine residue and at the carboxy terminus by a stop codon.

Many of the selected sequences do not consist of complete ORFs. Although a polypeptide representing a complete ORF may be the closest approximation of a protein native to an organism, it is not always preferred to express a complete ORF in a heterologous system. It may be challenging to express and purify a highly hydrophobic protein by common laboratory methods. Some of the polypeptide vaccine candidates described herein have been modified slightly to simplify the production of recombinant protein. For example, nucleotide sequences which encode highly hydrophobic domains, such as those found at the amino terminal signal sequence, have been excluded from some constructs used for *in vitro* expression of the polypeptides. Furthermore, any highly hydrophobic amino acid sequences occurring at the carboxy terminus have also been excluded from the recombinant expression constructs. Thus, in one embodiment, a polypeptide which represents a truncated or modified ORF may be used as an antigen.

While numerous methods are known in the art for selecting potentially immunogenic polypeptides, many of the ORFs disclosed herein were selected on the basis of screening all theoretical *Borrelia burgdorferi* ORFs for several aspects of potential immunogenicity. One set of selection criteria are as follows:

1. *Type I signal sequence:* An amino terminal type I signal sequence generally directs a nascent protein across the plasma and outer membranes to the exterior of the bacterial cell. Experimental evidence obtained from studies with *Escherichia coli* suggests that the typical type I signal sequence consists of the following biochemical and physical attributes (Izard, J. W. and Kendall, D. A. *Mol. Microbiol. 13*:765-773 (1994)). The length of the type I signal sequence is approximately 15 to 25 primarily hydrophobic amino acid residues with a net positive charge in the extreme amino terminus. In addition, the central region of the signal sequence adopts an alpha-helical conformation in a hydrophobic environment. Finally, the region surrounding the actual site of cleavage is ideally six residues long, with small side-chain amino acids in the -1 and -3 positions.

2. *Type IV signal sequence:* The type IV signal sequence is an example of the several types of functional signal sequences which exist in addition to the type I signal sequence detailed

above. Although functionally related, the type IV signal sequence possesses a unique set of biochemical and physical attributes (Strom, M. S. and Lory, S., *J. Bacteriol. 174*:7345-7351 (1992)). These are typically six to eight amino acids with a net basic charge followed by an additional sixteen to thirty primarily hydrophobic residues. The cleavage site of a type IV signal sequence is typically after the initial six to eight amino acids at the extreme amino terminus. In addition, type IV signal sequences generally contain a phenylalanine residue at the +1 site relative to the cleavage site.

3. *Lipoprotein:* Studies of the cleavage sites of twenty-six bacterial lipoprotein precursors has allowed the definition of a consensus amino acid sequence for lipoprotein cleavage. Nearly three-fourths of the bacterial lipoprotein precursors examined contained the sequence L-(A,S)-(G,A)-C at positions -3 to +1, relative to the point of cleavage (Hayashi, S. and Wu, H. C., *J. Bioenerg. Biomembr. 22*:451-471 (1990)).

4. *LPXTG motif:* It has been experimentally determined that most anchored proteins found on the surface of gram-positive bacteria possess a highly conserved carboxy terminal sequence. More than fifty such proteins from organisms such as *S. pyogenes*, *S. mutans*, *B. burgdorferi*, *S. pneumoniae*, and others, have been identified based on their extracellular location and carboxy terminal amino acid sequence (Fischetti, V. A., *ASM News 62*:405-410 (1996)). The conserved region consists of six charged amino acids at the extreme carboxy terminus coupled to 15-20 hydrophobic amino acids presumed to function as a transmembrane domain. Immediately adjacent to the transmembrane domain is a six amino acid sequence conserved in nearly all proteins examined. The amino acid sequence of this region is L-P-X-T-G-X, where X is any amino acid.

An algorithm for selecting antigenic and immunogenic *Borrelia burgdorferi* polypeptides including the foregoing criteria was developed. The algorithm is similar to that described in U.S. patent application 08/781,986, filed January 3, 1997, which is fully incorporated by reference herein. Use of the algorithm by the inventors to select immunologically useful *Borrelia burgdorferi* polypeptides resulted in the selection of a number of the disclosed ORFs. Polypeptides comprising the polypeptides identified in this group may be produced by techniques standard in the art and as further described herein.

Nucleic Acid Molecules

The present invention provides isolated nucleic acid molecules comprising polynucleotides encoding the *B. burgdorferi* polypeptides having the amino acid sequences shown in Table 1, which were determined by sequencing the genome of *B. burgdorferi* deposited as ATCC deposit no. 202012 and selected as putative immunogens.

Unless otherwise indicated, all nucleotide sequences determined by sequencing a DNA molecule herein were determined using an automated DNA sequencer (such as the Model 373 from Applied Biosystems, Inc.), and all amino acid sequences of polypeptides encoded by DNA molecules determined herein were predicted by translation of DNA sequences determined as

above. Therefore, as is known in the art for any DNA sequence determined by this automated approach, any nucleotide sequence determined herein may contain some errors. Nucleotide sequences determined by automation are typically at least about 90% identical, more typically at least about 95% to at least about 99.9% identical to the actual nucleotide sequence of the sequenced DNA molecule. The actual sequence can be more precisely determined by other approaches including manual DNA sequencing methods well known in the art. As is also known in the art, a single insertion or deletion in a determined nucleotide sequence compared to the actual sequence will cause a frame shift in translation of the nucleotide sequence such that the predicted amino acid sequence encoded by a determined nucleotide sequence will be completely different from the amino acid sequence actually encoded by the sequenced DNA molecule, beginning at the point of such an insertion or deletion.

Unless otherwise indicated, each "nucleotide sequence" set forth herein is presented as a sequence of deoxyribonucleotides (abbreviated A, G , C and T). However, by "nucleotide sequence" of a nucleic acid molecule or polynucleotide is intended, for a DNA molecule or polynucleotide, a sequence of deoxyribonucleotides, and for an RNA molecule or polynucleotide, the corresponding sequence of ribonucleotides (A, G, C and U), where each thymidine deoxyribonucleotide (T) in the specified deoxyribonucleotide sequence is replaced by the ribonucleotide uridine (U). For instance, reference to an RNA molecule having a sequence of Table 1 set forth using deoxyribonucleotide abbreviations is intended to indicate an RNA molecule having a sequence in which each deoxyribonucleotide A, G or C of Table 1 has been replaced by the corresponding ribonucleotide A, G or C, and each deoxyribonucleotide T has been replaced by a ribonucleotide U.

Nucleic acid molecules of the present invention may be in the form of RNA, such as mRNA, or in the form of DNA, including, for instance, cDNA and genomic DNA obtained by cloning or produced synthetically. The DNA may be double-stranded or single-stranded. Single-stranded DNA or RNA may be the coding strand, also known as the sense strand, or it may be the non-coding strand, also referred to as the anti-sense strand.

By "isolated" nucleic acid molecule(s) is intended a nucleic acid molecule, DNA or RNA, which has been removed from its native environment. For example, recombinant DNA molecules contained in a vector are considered isolated for the purposes of the present invention. Further examples of isolated DNA molecules include recombinant DNA molecules maintained in heterologous host cells or purified (partially or substantially) DNA molecules in solution. Isolated RNA molecules include *in vivo* or *in vitro* RNA transcripts of the DNA molecules of the present invention. Isolated nucleic acid molecules according to the present invention further include such molecules produced synthetically.

In addition, isolated nucleic acid molecules of the invention include DNA molecules which comprise a sequence substantially different from those described above but which, due to the degeneracy of the genetic code, still encode a *B. burgdorferi* polypeptides and peptides of the

the *B. burgdorferi* polypeptides of the present invention. This includes the genetic code and species-specific codon preferences known in the art. Thus, it would be routine for one skilled in the art to generate the degenerate variants described above, for instance, to optimize codon expression for a particular host (e.g., change codons in the bacteria mRNA to those preferred by a mammalian or other bacterial host such as *E. coli*).

The invention further provides isolated nucleic acid molecules having the nucleotide sequence shown in Table 1 or a nucleic acid molecule having a sequence complementary to one of the above sequences. Such isolated molecules, particularly DNA molecules, are useful as probes for gene mapping and for identifying *B. burgdorferi* in a biological sample, for instance, by PCR, Southern blot, Northern blot, or other form of hybridization analysis.

The present invention is further directed to nucleic acid molecules encoding portions or fragments of the nucleotide sequences described herein. Fragments include portions of the nucleotide sequences of Table 1 at least 10 contiguous nucleotides in length selected from any two integers, one of which representing a 5' nucleotide position and a second of which representing a 3' nucleotide position, where the first nucleotide for each nucleotide sequence in Table 1 is position 1. That is, every combination of a 5' and 3' nucleotide position that a fragment at least 10 contiguous nucleotides in length could occupy is included in the invention. "At least" means a fragment may be 10 contiguous nucleotide bases in length or any integer between 10 and the length of an entire nucleotide sequence of Table 1 minus 1. Therefore, included in the invention are contiguous fragments specified by any 5' and 3' nucleotide base positions of a nucleotide sequences of Table 1 wherein the contiguous fragment is any integer between 10 and the length of an entire nucleotide sequence minus 1.

Further, the invention includes polynucleotides comprising fragments specified by size, in nucleotides, rather than by nucleotide positions. The invention includes any fragment size, in contiguous nucleotides, selected from integers between 10 and the length of an entire nucleotide sequence minus 1. Preferred sizes of contiguous nucleotide fragments include 20 nucleotides, 30 nucleotides, 40 nucleotides, 50 nucleotides. Other preferred sizes of contiguous nucleotide fragments, which may be useful as diagnostic probes and primers, include fragments 50-300 nucleotides in length which include, as discussed above, fragment sizes representing each integer between 50-300. Larger fragments are also useful according to the present invention corresponding to most, if not all, of the nucleotide sequences shown in Table 1or of the *B. burgdorferi* nucleotide sequences of the plasimd clones listed in Table 1. The preferred sizes are, of course, meant to exemplify not limit the present invention as all size fragments, representing any integer between 10 and the length of an entire nucleotide sequence minus 1, are included in the invention. Additional preferred nucleic acid fragments of the present invention include nucleic acid molecules encoding epitope-bearing portions of *B.*

Table 1 or the plasimd clones listed in Table 1. Any number of fragments of nucleotide sequences in Table 1 or the plasimd clones listed in Table 1, specified by 5' and 3' base positions or by size in nucleotides, as described above, may be excluded from the present invention.

Preferred nucleic acid fragments of the present invention also include nucleic acid molecules encoding epitope-bearing portions of the *B. burgdorferi* polypeptides shown in Table 1. Such nucleic acid fragments of the present invention include, for example, nucleic acid molecules encoding polypeptide fragments comprising from about the amino terminal residue to about the carboxy terminal residue of each fragment shown in Table 4. The above referred to polypeptide fragments are antigenic regions of particular *B. burgdorferi* polypeptides shown in Table 1. Methods for determining other such epitope-bearing portions for the remaining polypeptides described in Table 1 are well known in the art and are described in detail below.

In another aspect, the invention provides isolated nucleic acid molecules comprising polynucleotides which hybridize under stringent hybridization conditions to a portion of a polynucleotide in a nucleic acid molecule of the invention described above, for instance, a nucleic acid sequence shown in Table 1. By "stringent hybridization conditions" is intended overnight incubation at 42 C in a solution comprising: 50% formamide, 5x SSC (150 mM NaCl, 15 mM trisodium citrate), 50 mM sodium phosphate (pH 7.6), 5x Denhardt's solution, 10% dextran sulfate, and 20 g/ml denatured, sheared salmon sperm DNA, followed by washing the filters in 0.1x SSC at about 65 C.

By polynucleotides which hybridize to a "portion" of a polynucleotide is intended polynucleotides (either DNA or RNA) which hybridize to at least about 15 nucleotides (nt), and more preferably at least about 20 nt, still more preferably at least about 30 nt, and even more preferably about 30-70 nt of the reference polynucleotide. These are useful as diagnostic probes and primers as discussed above and in more detail below.

Of course, polynucleotides hybridizing to a larger portion of the reference polynucleotide, for instance, a portion 50-100 nt in length, or even to the entire length of the reference polynucleotide, are also useful as probes according to the present invention, as are polynucleotides corresponding to most, if not all, of a nucleotide sequence as shown in Table 1. By a portion of a polynucleotide of "at least 20 nt in length," for example, is intended 20 or more contiguous nucleotides from the nucleotide sequence of the reference polynucleotide (*e.g.*, a nucleotide sequences as shown in Table 1). As noted above, such portions are useful diagnostically either as probes according to conventional DNA hybridization techniques or as primers for amplification of a target sequence by PCR, as described, for instance, in *Molecular Cloning, A Laboratory Manual*, 2nd. edition, Sambrook, J., Fritsch, E. F. and Maniatis, T., eds., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. (1989), the entire disclosure of which is hereby incorporated herein by reference.

Since nucleic acid sequences encoding the *B. burgdorferi* polypeptides of the present

of the present invention could be generated synthetically according to known techniques.

As indicated, nucleic acid molecules of the present invention which encode *B. burgdorferi* polypeptides of the present invention may include, but are not limited to those encoding the amino acid sequences of the polypeptides by themselves; and additional coding sequences which code for additional amino acids, such as those which provide additional functionalities. Thus, the sequences encoding these polypeptides may be fused to a marker sequence, such as a sequence encoding a peptide which facilitates purification of the fused polypeptide. In certain preferred embodiments of this aspect of the invention, the marker amino acid sequence is a hexa-histidine peptide, such as the tag provided in a pQE vector (Qiagen, Inc.), among others, many of which are commercially available. As described in Gentz *et al., Proc. Natl. Acad. Sci. USA 86*:821-824 (1989), for instance, hexa-histidine provides for convenient purification of the resulting fusion protein.

Thus, the present invention also includes genetic fusions wherein the *B. burgdorferi* nucleic acid sequences coding sequences provided in Table 1 are linked to additional nucleic acid sequences to produce fusion proteins. These fusion proteins may include epitopes of borrelial or non-borrelial origin designed to produce proteins having enhanced immunogenicity. Further, the fusion proteins of the present invention may contain antigenic determinants known to provide helper T-cell stimulation, peptides encoding sites for post-translational modifications which enhance immunogenicity (*e.g.*, acylation), peptides which facilitate purification (*e.g.*, histidine "tag"), or amino acid sequences which target the fusion protein to a desired location (*e.g.*, a heterologous leader sequence). For instance, hexa-histidine provides for convenient purification of the fusion protein. See Gentz et al. (1989) Proc. Natl. Acad. Sci. 86:821-24. The "HA" tag is another peptide useful for purification which corresponds to an epitope derived from the influenza hemagglutinin protein. See Wilson et al. (1984) Cell 37:767. As discussed below, other such fusion proteins include the *B. burgdorferi* polypeptides of the present invention fused to Fc at the N- or C-terminus.

Post-translational modification of the full-length *B. burgdorferi* OspA protein expressed in *E. coli* is believed to increase the immunogenicity of this protein. Erdile, L. *et al., Infect. Immun. 61*:81-90 (1993). *B. burgdorferi* OspA when expressed in *E. coli*, for example, is post-translationally modified in at least two ways. First, a signal peptide is cleaved; second, lipid moieties are attached. The presence of these lipid moieties is believed to confer enhanced immunogenicity and results in the elicitation of a strong protective immunological response.

Variant and Mutant Polynucleotides

The present invention thus includes nucleic acid molecules and sequences which encode fusion proteins comprising one or more *B. burgdorferi* polypeptides of the present invention fused to an amino acid sequence which allows for post-translational modification to enhance

of an amino acid sequence which results in the attachment of a lipid moiety. Such a lipid moiety attachment site of OspA, which is lipidated upon expression in *E. coli*, has been identified. Bouchon, B. *et al., Anal. Biochem. 246*:52-61 (1997).

Thus, as indicated above, the present invention includes genetic fusions wherein a *B. burgdorferi* nucleic acid sequence provided in Table 1 is linked to a nucleotide sequence encoding another amino acid sequence. These other amino acid sequences may be of borrelial origin (*e.g.*, another sequence selected from Table 1) or non-borrelial origin. An example of such a fusion protein is reported in Fikrig, E. *et al., Science 250*:553-556 (1990) where an OspA-glutathione-*S*-transferase fusion protein was produced and shown to elicit protective immunity against Lyme disease in immune competent mice.

The present invention further relates to variants of the nucleic acid molecules of the present invention, which encode portions, analogs or derivatives of the *B. burgdorferi* polypeptides shown in Table 1. Variants may occur naturally, such as a natural allelic variant. By an "allelic variant" is intended one of several alternate forms of a gene occupying a given locus on a chromosome of an organism. *Genes II*, Lewin, B., ed., John Wiley & Sons, New York (1985). Non-naturally occurring variants may be produced using art-known mutagenesis techniques.

Such variants include those produced by nucleotide substitutions, deletions or additions. The substitutions, deletions or additions may involve one or more nucleotides. These variants may be altered in coding regions, non-coding regions, or both. Alterations in the coding regions may produce conservative or non-conservative amino acid substitutions, deletions or additions. Especially preferred among these are silent substitutions, additions and deletions, which do not alter the properties and activities of the *B. burgdorferi* polypeptides disclosed herein or portions thereof. Also especially preferred in this regard are conservative substitutions.

The present application is further directed to nucleic acid molecules at least 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleic acid sequence shown in Table 1. The above nucleic acid sequences are included irrespective of whether they encode a polypeptide having *B. burgdorferi* activity. This is because even where a particular nucleic acid molecule does not encode a polypeptide having *B. burgdorferi* activity, one of skill in the art would still know how to use the nucleic acid molecule, for instance, as a hybridization probe. Uses of the nucleic acid molecules of the present invention that do not encode a polypeptide having *B. burgdorferi* activity include, *inter alia*, isolating an *B. burgdorferi* gene or allelic variants thereof from a DNA library, and detecting *B. burgdorferi* mRNA expression samples, environmental samples, suspected of containing *B. burgdorferi* by Northern Blot analysis.

Embodiments of the invention include isolated nucleic acid molecules comprising a polynucleotide having a nucleotide sequence at least 90% identical, and more preferably at least 95%, 96%, 97%, 98% or 99% identical to (a) a nucleotide sequence encoding any of the amino acid sequences of the full-length polypeptides shown in Table 1; (b) a nucleotide sequence

amino acid sequences of the truncated polypeptides shown in Table 1; and (d) a nucleotide sequence complementary to any of the nucleotide sequences in (a), (b), or (c) above.

Preferred, are nucleic acid molecules having sequences at least 90%, 95%, 96%, 97%, 98% or 99% identical to the nucleic acid sequence shown in Table 1, which do, in fact, encode a polypeptide having *B. burgdorferi* protein activity By "a polypeptide having *B. burgdorferi* activity" is intended polypeptides exhibiting activity similar, but not necessarily identical, to an activity of the *B. burgdorferi* protein of the invention, as measured in a particular biological assay suitable for measuring activity of the specified protein.

Due to the degeneracy of the genetic code, one of ordinary skill in the art will immediately recognize that a large number of the nucleic acid molecules having a sequence at least 90%, 95%, 96%, 97%, 98%, or 99% identical to the nucleic acid sequences shown in Table 1 will encode a polypeptide having *B. burgdorferi* protein activity. In fact, since degenerate variants of these nucleotide sequences all encode the same polypeptide, this will be clear to the skilled artisan even without performing the above described comparison assay. It will be further recognized in the art that, for such nucleic acid molecules that are not degenerate variants, a reasonable number will also encode a polypeptide having *B. burgdorferi* protein activity. This is because the skilled artisan is fully aware of amino acid substitutions that are either less likely or not likely to significantly effect protein function (e.g., replacing one aliphatic amino acid with a second aliphatic amino acid), as further described below.

The biological activity or function of the polypeptides of the present invention are expected to be similar or identical to polypeptides from other bacteria that share a high degree of structural identity/similarity. Tables 2 lists accession numbers and descriptions for the closest matching sequences of polypeptides available through Genbank and Derwent databases. It is therefore expected that the biological activity or function of the polypeptides of the present invention will be similar or identical to those polypeptides from other bacterial genuses, species, or strains listed in Table 2.

By a polynucleotide having a nucleotide sequence at least, for example, 95% "identical" to a reference nucleotide sequence of the present invention, it is intended that the nucleotide sequence of the polynucleotide is identical to the reference sequence except that the polynucleotide sequence may include up to five point mutations per each 100 nucleotides of the reference nucleotide sequence encoding the *B. burgdorferi* polypeptide. In other words, to obtain a polynucleotide having a nucleotide sequence at least 95% identical to a reference nucleotide sequence, up to 5% (5 of 100) of the nucleotides in the reference sequence may be deleted, inserted, or substituted with another nucleotide. The query sequence may be an entire sequence shown in Table 1, the ORF (open reading frame), or any fragment specified as described herein.

As a practical matter, whether any particular nucleic acid molecule or polypeptide is at least

and a subject sequence, also referred to as a global sequence alignment, can be determined using the FASTDB computer program based on the algorithm of Brutlag et al. *See* Brutlag et al. (1990) Comp. App. Biosci. 6:237-245. In a sequence alignment the query and subject sequences are both DNA sequences. An RNA sequence can be compared by first converting U's to T's. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB alignment of DNA sequences to calculate percent identity are: Matrix=Unitary, k-tuple=4, Mismatch Penalty=1, Joining Penalty=30, Randomization Group Length=0, Cutoff Score=1, Gap Penalty=5, Gap Size Penalty 0.05, Window Size=500 or the lenght of the subject nucleotide sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence because of 5' or 3' deletions, not because of internal deletions, a manual correction must be made to the results. This is because the FASTDB program does not account for 5' and 3' truncations of the subject sequence when calculating percent identity. For subject sequences truncated at the 5' or 3' ends, relative to the query sequence, the percent identity is corrected by calculating the number of bases of the query sequence that are 5' and 3' of the subject sequence, which are not matched/aligned, as a percent of the total bases of the query sequence. Whether a nucleotide is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This corrected score is what is used for the purposes of the present invention. Only nucleotides outside the 5' and 3' nucleotides of the subject sequence, as displayed by the FASTDB alignment, which are not matched/aligned with the query sequence, are calculated for the purposes of manually adjusting the percent identity score.

For example, a 90 nucleotide subject sequence is aligned to a 100 nucleotide query sequence to determine percent identity. The deletions occur at the 5' end of the subject sequence and therefore, the FASTDB alignment does not show a matched/alignment of the first 10 nucleotides at 5' end. The 10 unpaired nucleotides represent 10% of the sequence (number of nucleotides at the 5' and 3' ends not matched/total number of nucleotides in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 nucleotides were perfectly matched the final percent identity would be 90%. In another example, a 90 nucleotide subject sequence is compared with a 100 nucleotide query sequence. This time the deletions are internal deletions so that there are no nucleotides on the 5' or 3' of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only nucleotides 5' and 3' of the subject sequence which are not matched/aligned with the query sequence are manually corrected for. No other manual corrections are to made for the purposes of the present invention.

Vectors and Host Cells

the present invention, host cells which are genetically engineered with the recombinant vectors, and the production of *B. burgdorferi* polypeptides or fragments thereof by recombinant techniques.

Recombinant constructs may be introduced into host cells using well known techniques such as infection, transduction, transfection, transvection, electroporation and transformation. The vector may be, for example, a phage, plasmid, viral or retroviral vector. Retroviral vectors may be replication competent or replication defective. In the latter case, viral propagation generally will occur only in complementing host cells.

The polynucleotides may be joined to a vector containing a selectable marker for propagation in a host. Generally, a plasmid vector is introduced in a precipitate, such as a calcium phosphate precipitate, or in a complex with a charged lipid. If the vector is a virus, it may be packaged *in vitro* using an appropriate packaging cell line and then transduced into host cells.

Preferred are vectors comprising *cis*-acting control regions to the polynucleotide of interest. Appropriate *trans*-acting factors may be supplied by the host, supplied by a complementing vector or supplied by the vector itself upon introduction into the host.

In certain preferred embodiments in this regard, the vectors provide for specific expression, which may be inducible and/or cell type-specific. Particularly preferred among such vectors are those inducible by environmental factors that are easy to manipulate, such as temperature and nutrient additives.

Expression vectors useful in the present invention include chromosomal-, episomal- and virus-derived vectors, *e.g.*, vectors derived from bacterial plasmids, bacteriophage, yeast episomes, yeast chromosomal elements, viruses such as baculoviruses, papova viruses, vaccinia viruses, adenoviruses, fowl pox viruses, pseudorabies viruses and retroviruses, and vectors derived from combinations thereof, such as cosmids and phagemids.

The DNA insert should be operatively linked to an appropriate promoter, such as the phage lambda PL promoter, the *E. coli lac, trp* and *tac* promoters, the SV40 early and late promoters and promoters of retroviral LTRs, to name a few. Other suitable promoters will be known to the skilled artisan. The expression constructs will further contain sites for transcription initiation, termination and, in the transcribed region, a ribosome binding site for translation. The coding portion of the mature transcripts expressed by the constructs will preferably include a translation initiating site at the beginning and a termination codon (UAA, UGA or UAG) appropriately positioned at the end of the polypeptide to be translated.

As indicated, the expression vectors will preferably include at least one selectable marker. Such markers include dihydrofolate reductase or neomycin resistance for eukaryotic cell culture and tetracycline or ampicillin resistance genes for culturing in *E. coli* and other bacteria. Representative examples of appropriate hosts include, but are not limited to, bacterial cells, such as *E. coli*, *Streptomyces* and *Salmonella typhimurium* cells; fungal cells, such as yeast cells; insect cells such as *Drosophila* S2 and *Spodoptera* Sf9 cells; animal cells such as CHO, COS and

above-described host cells are known in the art.

Among vectors preferred for use in bacteria include pQE70, pQE60 and pQE-9, available from Qiagen; pBS vectors, Phagescript vectors, Bluescript vectors, pNH8A, pNH16a, pNH18A, pNH46A available from Stratagene; pET series of vectors available from Novagen; and ptrc99a, pKK223-3, pKK233-3, pDR540, pRIT5 available from Pharmacia. Among preferred eukaryotic vectors are pWLNEO, pSV2CAT, pOG44, pXT1 and pSG available from Stratagene; and pSVK3, pBPV, pMSG and pSVL available from Pharmacia. Other suitable vectors will be readily apparent to the skilled artisan.

Among known bacterial promoters suitable for use in the present invention include the *E. coli lac*I and *lac*Z promoters, the T3 and T7 promoters, the *gpt* promoter, the lambda PR and PL promoters and the *trp* promoter. Suitable eukaryotic promoters include the CMV immediate early promoter, the HSV thymidine kinase promoter, the early and late SV40 promoters, the promoters of retroviral LTRs, such as those of the Rous sarcoma virus (RSV), and metallothionein promoters, such as the mouse metallothionein-I promoter.

Introduction of the construct into the host cell can be effected by calcium phosphate transfection, DEAE-dextran mediated transfection, cationic lipid-mediated transfection, electroporation, transduction, infection or other methods. Such methods are described in many standard laboratory manuals, such as Davis *et al., Basic Methods In Molecular Biology* (1986).

Transcription of DNA encoding the polypeptides of the present invention by higher eukaryotes may be increased by inserting an enhancer sequence into the vector. Enhancers are *cis*-acting elements of DNA, usually about from 10 to 300 bp that act to increase transcriptional activity of a promoter in a given host cell-type. Examples of enhancers include the SV40 enhancer, which is located on the late side of the replication origin at bp 100 to 270, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers.

For secretion of the translated polypeptide into the lumen of the endoplasmic reticulum, into the periplasmic space or into the extracellular environment, appropriate secretion signals may be incorporated into the expressed polypeptide. The signals may be endogenous to the polypeptide or they may be heterologous signals.

The polypeptide may be expressed in a modified form, such as a fusion protein, and may include not only secretion signals, but also additional heterologous functional regions. For instance, a region of additional amino acids, particularly charged amino acids, may be added to the N-terminus of the polypeptide to improve stability and persistence in the host cell, during purification, or during subsequent handling and storage. Also, peptide moieties may be added to the polypeptide to facilitate purification. Such regions may be removed prior to final preparation of the polypeptide. The addition of peptide moieties to polypeptides to engender secretion or excretion, to improve stability and to facilitate purification, among others, are familiar and routine techniques in the art. A preferred fusion protein comprises a heterologous region from

counterpart 2045869) discloses fusion proteins comprising various portions of constant region of immunoglobin molecules together with another human protein or part thereof. In many cases, the Fc part in a fusion protein is thoroughly advantageous for use in therapy and diagnosis and thus results, for example, in improved pharmacokinetic properties (EP-A 0232 262). On the other hand, for some uses it would be desirable to be able to delete the Fc part after the fusion protein has been expressed, detected and purified in the advantageous manner described. This is the case when Fc portion proves to be a hindrance to use in therapy and diagnosis, for example when the fusion protein is to be used as antigen for immunizations. In drug discovery, for example, human proteins, such as, hIL5-receptor has been fused with Fc portions for the purpose of high-throughput screening assays to identify antagonists of hIL-5. *See* Bennett, D. *et al.*, *J. Molec. Recogn. 8*:52-58 (1995) and Johanson, K. *et al.*, *J. Biol. Chem. 270 (16)*:9459-9471 (1995).

The *B. burgdorferi* polypeptides can be recovered and purified from recombinant cell cultures by well-known methods including ammonium sulfate or ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction chromatography, affinity chromatography, hydroxylapatite chromatography, lectin chromatography and high performance liquid chromatography ("HPLC") is employed for purification. Polypeptides of the present invention include naturally purified products, products of chemical synthetic procedures, and products produced by recombinant techniques from a prokaryotic or eukaryotic host, including, for example, bacterial, yeast, higher plant, insect and mammalian cells.

Polypeptides and Fragments

The invention further provides isolated polypeptides having the amino acid sequences in Table 1, and peptides or polypeptides comprising portions of the above polypeptides. The terms "peptide" and "oligopeptide" are considered synonymous (as is commonly recognized) and each term can be used interchangeably as the context requires to indicate a chain of at least to amino acids coupled by peptidyl linkages. The word "polypeptide" is used herein for chains containing more than ten amino acid residues. All oligopeptide and polypeptide formulas or sequences herein are written from left to right and in the direction from amino terminus to carboxy terminus.

As discussed in detail below, immunization using *B. burgdorferi* sensu stricto isolate B31 decorin-binding protein elicits the production of antiserum which confers passive immunity against *Borrelia* species and strains which express divergent forms of this protein. Cassatt, D. *et al., Protection of Borrelia burgdorferi Infection by Antibodies to Decorin-binding Protein*, in VACCINES97, Cold Spring Harbor Press (1997), pages 191-195. Thus, some amino acid sequences of the *B. burgdorferi* polypeptides shown in Table 1 can be varied without significantly effecting the antigenicity of the polypeptides. If such differences in sequence are

antigenic epitope without significantly effecting the antigenicity of a polypeptide.

Variant and Mutant Polypeptides

To improve or alter the characteristics of *B. burgdorferi* polypeptides of the present invention, protein engineering may be employed. Recombinant DNA technology known to those skilled in the art can be used to create novel mutant proteins or muteins including single or multiple amino acid substitutions, deletions, additions, or fusion proteins. Such modified polypeptides can show, e.g., enhanced activity or increased stability. In addition, they may be purified in higher yields and show better solubility than the corresponding natural polypeptide, at least under certain purification and storage conditions.

N-Terminal and C-Terminal Deletion Mutants

It is known in the art that one or more amino acids may be deleted from the N-terminus or C-terminus without substantial loss of biological function. For instance, Ron et al. J. Biol. Chem., 268:2984-2988 (1993), reported modified KGF proteins that had heparin binding activity even if 3, 8, or 27 N-terminal amino acid residues were missing. Accordingly, the present invention provides polypeptides having one or more residues deleted from the amino terminus of the amino acid sequence of the *B. burgdorferi* polypeptides shown in Table 1, and polynucleotides encoding such polypeptides.

Similarly, many examples of biologically functional C-terminal deletion muteins are known. For instance, Interferon gamma shows up to ten times higher activities by deleting 8-10 amino acid residues from the carboxy terminus of the protein *See, e.g.*, Dobeli, et al. (1988) J. Biotechnology 7:199-216. Accordingly, the present invention provides polypeptides having one or more residues from the carboxy terminus of the amino acid sequence of the *B. burgdorferi* polypeptides shown in Table 1. The invention also provides polypeptides having one or more amino acids deleted from both the amino and the carboxyl termini as described below.

The present invention is further directed to polynucleotide encoding portions or fragments of the amino acid sequences described herein as well as to portions or fragments of the isolated amino acid sequences described herein. Fragments include portions of the amino acid sequences of Table 1, are at least 5 contiguous amino acid in length, are selected from any two integers, one of which representing a N-terminal position. The initiation codon of the polypeptides of the present inventions position 1. Every combination of a N-terminal and C-terminal position that a fragment at least 5 contiguous amino acid residues in length could occupy, on any given amino acid sequence of Table 1 is included in the invention. At least means a fragment may be 5 contiguous amino acid residues in length or any integer between 5 and the number of residues in a full length amino acid sequence minus 1. Therefore, included in the invention are contiguous fragments specified by any N-terminal and C-terminal positions of amino acid sequence set forth in Table 1 wherein the contiguous fragment is any integer between 5 and the number of residues in a full length sequence minus 1.

Further, the invention includes polypeptides comprising fragments specified by size, in

amino acid residues, rather than by N-terminal and C-terminal positions. The invention includes any fragment size, in contiguous amino acid residues, selected from integers between 5 and the number of residues in a full length sequence minus 1. Preferred sizes of contiguous polypeptide fragments include about 5 amino acid residues, about 10 amino acid residues, about 20 amino acid residues, about 30 amino acid residues, about 40 amino acid residues, about 50 amino acid residues, about 100 amino acid residues, about 200 amino acid residues, about 300 amino acid residues, and about 400 amino acid residues. The preferred sizes are, of course, meant to exemplify, not limit, the present invention as all size fragments representing any integer between 5 and the number of residues in a full length sequence minus 1 are included in the invention. The present invention also provides for the exclusion of any fragments specified by N-terminal and C-terminal positions or by size in amino acid residues as described above. Any number of fragments specified by N-terminal and C-terminal positions or by size in amino acid residues as described above may be excluded.

The above fragments need not be active since they would be useful, for example, in immunoassays, in epitope mapping, epitope tagging, to generate antibodies to a particular portion of the protein, as vaccines, and as molecular weight markers.

Other Mutants

In addition to N- and C-terminal deletion forms of the protein discussed above, it also will be recognized by one of ordinary skill in the art that some amino acid sequences of the *B. burgdorferi* polypeptide can be varied without significant effect of the structure or function of the protein. If such differences in sequence are contemplated, it should be remembered that there will be critical areas on the protein which determine activity.

Thus, the invention further includes variations of the *B. burgdorferi* polypeptides which show substantial *B. burgdorferi* polypeptide activity or which include regions of *B. burgdorferi* protein such as the protein portions discussed below. Such mutants include deletions, insertions, inversions, repeats, and type substitutions selected according to general rules known in the art so as to have little effect on activity. For example, guidance concerning how to make phenotypically silent amino acid substitutions is provided. There are two main approaches for studying the tolerance of an amino acid sequence to change. *See*, Bowie, J. U. *et al.* (1990), Science 247:1306-1310. The first method relies on the process of evolution, in which mutations are either accepted or rejected by natural selection. The second approach uses genetic engineering to introduce amino acid changes at specific positions of a cloned gene and selections or screens to identify sequences that maintain functionality.

These studies have revealed that proteins are surprisingly tolerant of amino acid substitutions. The studies indicate which amino acid changes are likely to be permissive at a certain position of the protein. For example, most buried amino acid residues require nonpolar side chains, whereas few features of surface side chains are generally conserved. Other such

therein. Typically seen as conservative substitutions are the replacements, one for another, among the aliphatic amino acids Ala, Val, Leu and Ile; interchange of the hydroxyl residues Ser and Thr, exchange of the acidic residues Asp and Glu, substitution between the amide residues Asn and Gln, exchange of the basic residues Lys and Arg and replacements among the aromatic residues Phe, Tyr.

Thus, the fragment, derivative, analog, or homolog of the polypeptide of Table 1, or that encoded by the plaimds listed in Table 1, may be: (i) one in which one or more of the amino acid residues are substituted with a conserved or non-conserved amino acid residue (preferably a conserved amino acid residue) and such substituted amino acid residue may or may not be one encoded by the genetic code: or (ii) one in which one or more of the amino acid residues includes a substituent group: or (iii) one in which the *B. burgdorferi* polypeptide is fused with another compound, such as a compound to increase the half-life of the polypeptide (for example, polyethylene glycol): or (iv) one in which the additional amino acids are fused to the above form of the polypeptide, such as an IgG Fc fusion region peptide or leader or secretory sequence or a sequence which is employed for purification of the above form of the polypeptide or a proprotein sequence. Such fragments, derivatives and analogs are deemed to be within the scope of those skilled in the art from the teachings herein.

Thus, the *B. burgdorferi* polypeptides of the present invention may include one or more amino acid substitutions, deletions, or additions, either from natural mutations or human manipulation. As indicated, changes are preferably of a minor nature, such as conservative amino acid substitutions that do not significantly affect the folding or activity of the protein (see Table 3).

Amino acids in the *B. burgdorferi* proteins of the present invention that are essential for function can be identified by methods known in the art, such as site-directed mutagenesis or alanine-scanning mutagenesis. *See, e.g.*, Cunningham et al. (1989) Science 244:1081-1085. The latter procedure introduces single alanine mutations at every residue in the molecule. The resulting mutant molecules are then tested for biological activity using assays appropriate for measuring the function of the particular protein.

Of special interest are substitutions of charged amino acids with other charged or neutral amino acids which may produce proteins with highly desirable improved characteristics, such as less aggregation. Aggregation may not only reduce activity but also be problematic when preparing pharmaceutical formulations, because aggregates can be immunogenic. *See, e.g.*, Pinckard et al., (1967) Clin. Exp. Immunol. 2:331-340; Robbins, et al., (1987) Diabetes 36:838-845; Cleland, et al., (1993) Crit. Rev. Therapeutic Drug Carrier Systems 10:307-377.

The polypeptides of the present invention are preferably provided in an isolated form, and preferably are substantially purified. A recombinantly produced version of the *B. burgdorferi* polypeptide can be substantially purified by the one-step method described by Smith et al. (1988) Gene 67:31-40. Polypeptides of the invention also can be purified from natural or recombinant

The invention further provides for isolated *B. burgdorferi* polypeptides comprising an amino acid sequence selected from the group consisting of: (a) the amino acid sequence of a full-length *B. burgdorferi* polypeptide having the complete amino acid sequence shown in Table 1; (b) the amino acid sequence of a full-length *B. burgdorferi* polypeptide having the complete amino acid sequence shown in Table 1 excepting the N-terminal methionine; (c) the complete amino acid sequence encoded by the plaimds listed in Table 1; and (d) the complete amino acid sequence excepting the N-terminal methionine encoded by the plaimds listed in Table 1. The polypeptides of the present invention also include polypeptides having an amino acid sequence at least 80% identical, more preferably at least 90% identical, and still more preferably 95%, 96%, 97%, 98% or 99% identical to those described in (a), (b), (c), and (d) above.

Further polypeptides of the present invention include polypeptides which have at least 90% similarity, more preferably at least 95% similarity, and still more preferably at least 96%, 97%, 98% or 99% similarity to those described above.

A further embodiment of the invention relates to a polypeptide which comprises the amino acid sequence of a *B. burgdorferi* polypeptide having an amino acid sequence which contains at least one conservative amino acid substitution, but not more than 50 conservative amino acid substitutions, not more than 40 conservative amino acid substitutions, not more than 30 conservative amino acid substitutions, and not more than 20 conservative amino acid substitutions. Also provided are polypeptides which comprise the amino acid sequence of a *B. burgdorferi* polypeptide, having at least one, but not more than 10, 9, 8, 7, 6, 5, 4, 3, 2 or 1 conservative amino acid substitutions.

By a polypeptide having an amino acid sequence at least, for example, 95% "identical" to a query amino acid sequence of the present invention, it is intended that the amino acid sequence of the subject polypeptide is identical to the query sequence except that the subject polypeptide sequence may include up to five amino acid alterations per each 100 amino acids of the query amino acid sequence. In other words, to obtain a polypeptide having an amino acid sequence at least 95% identical to a query amino acid sequence, up to 5% of the amino acid residues in the subject sequence may be inserted, deleted, (indels) or substituted with another amino acid. These alterations of the reference sequence may occur at the amino or carboxy terminal positions of the reference amino acid sequence or anywhere between those terminal positions, interspersed either individually among residues in the reference sequence or in one or more contiguous groups within the reference sequence.

As a practical matter, whether any particular polypeptide is at least 90%, 95%, 96%, 97%, 98% or 99% identical to, for instance, the amino acid sequences shown in Table 1 or to the amino acid sequence encoded by the plaimds listed in Table 1 can be determined conventionally using known computer programs. A preferred method for determining the best overall match between a

query and subject sequences are both amino acid sequences. The result of said global sequence alignment is in percent identity. Preferred parameters used in a FASTDB amino acid alignment are: Matrix=PAM 0, k-tuple=2, Mismatch Penalty=1, Joining Penalty=20, Randomization Group Length=0, Cutoff Score=1, Window Size=sequence length, Gap Penalty=5, Gap Size Penalty=0.05, Window Size=500 or the length of the subject amino acid sequence, whichever is shorter.

If the subject sequence is shorter than the query sequence due to N- or C-terminal deletions, not because of internal deletions, the results, in percent identity, must be manually corrected. This is because the FASTDB program does not account for N- and C-terminal truncations of the subject sequence when calculating global percent identity. For subject sequences truncated at the N- and C-termini, relative to the query sequence, the percent identity is corrected by calculating the number of residues of the query sequence that are N- and C-terminal of the subject sequence, which are not matched/aligned with a corresponding subject residue, as a percent of the total bases of the query sequence. Whether a residue is matched/aligned is determined by results of the FASTDB sequence alignment. This percentage is then subtracted from the percent identity, calculated by the above FASTDB program using the specified parameters, to arrive at a final percent identity score. This final percent identity score is what is used for the purposes of the present invention. Only residues to the N- and C-termini of the subject sequence, which are not matched/aligned with the query sequence, are considered for the purposes of manually adjusting the percent identity score. That is, only query amino acid residues outside the farthest N- and C-terminal residues of the subject sequence.

For example, a 90 amino acid residue subject sequence is aligned with a 100 residue query sequence to determine percent identity. The deletion occurs at the N-terminus of the subject sequence and therefore, the FASTDB alignment does not match/align with the first 10 residues at the N-terminus. The 10 unpaired residues represent 10% of the sequence (number of residues at the N- and C- termini not matched/total number of residues in the query sequence) so 10% is subtracted from the percent identity score calculated by the FASTDB program. If the remaining 90 residues were perfectly matched the final percent identity would be 90%. In another example, a 90 residue subject sequence is compared with a 100 residue query sequence. This time the deletions are internal so there are no residues at the N- or C-termini of the subject sequence which are not matched/aligned with the query. In this case the percent identity calculated by FASTDB is not manually corrected. Once again, only residue positions outside the N- and C-terminal ends of the subject sequence, as displayed in the FASTDB alignment, which are not matched/aligned with the query sequence are manually corrected. No other manual corrections are to made for the purposes of the present invention.

The above polypeptide sequences are included irrespective of whether they have their normal biological activity. This is because even where a particular polypeptide molecule does not

invention that do not have *B. burgdorferi* activity include, *inter alia*, as epitope tags, in epitope mapping, and as molecular weight markers on SDS-PAGE gels or on molecular sieve gel filtration columns using methods known to those of skill in the art.

As described below, the polypeptides of the present invention can also be used to raise polyclonal and monoclonal antibodies, which are useful in assays for detecting *B. burgdorferi* protein expression or as agonists and antagonists capable of enhancing or inhibiting *B. burgdorferi* protein function. Further, such polypeptides can be used in the yeast two-hybrid system to "capture" *B. burgdorferi* protein binding proteins which are also candidate agonists and antagonists according to the present invention. *See, e.g.,* Fields et al. (1989) Nature 340:245-246.

Epitope-Bearing Portions

In another aspect, the invention provides peptides and polypeptides comprising epitope-bearing portions of the *B. burgdorferi* polypeptides of the present invention. These epitopes are immunogenic or antigenic epitopes of the polypeptides of the present invention. An "immunogenic epitope" is defined as a part of a protein that elicits an antibody response when the whole protein or polypeptide is the immunogen. These immunogenic epitopes are believed to be confined to a few loci on the molecule. On the other hand, a region of a protein molecule to which an antibody can bind is defined as an "antigenic determinant" or "antigenic epitope." The number of immunogenic epitopes of a protein generally is less than the number of antigenic epitopes. *See, e.g.,* Geysen, et al. (1983) Proc. Natl. Acad. Sci. USA 81:3998- 4002. Predicted antigenic epitopes are shown in Table 4, below. It is pointed out that Table 4 only lists amino acid residues comprising epitopes predicted to have the highest degree of antigenicity. The polypeptides not listed in Table 4 and portions of polypeptides not listed in Table 4 are not considered non-antigenic. This is because they may still be antigenic *in vivo* but merely not recognized as such by the particular algorithm used. Thus, Table 4 lists the amino acid residues comprising preferred antigenic epitopes but not a complete list. Amino acid residues comprising other anigenic epitopes may be determined by algorithms similar to the Jameson-Wolf analysis or by *in vivo* testing for an antigenic response using the methods described herein or those known in the art.

As to the selection of peptides or polypeptides bearing an antigenic epitope (*i.e.*, that contain a region of a protein molecule to which an antibody can bind), it is well known in that art that relatively short synthetic peptides that mimic part of a protein sequence are routinely capable of eliciting an antiserum that reacts with the partially mimicked protein. *See, e.g.,* Sutcliffe, et al., (1983) Science 219:660-666. Peptides capable of eliciting protein-reactive sera are frequently represented in the primary sequence of a protein, can be characterized by a set of simple chemical rules, and are confined neither to immunodominant regions of intact proteins (*i.e.*, immunogenic epitopes) nor to the amino or carboxyl terminals. Peptides that are extremely hydrophobic and

mimicked protein; longer, peptides, especially those containing proline residues, usually are effective. *See,* Sutcliffe, et al., *supra*, p. 661. For instance, 18 of 20 peptides designed according to these guidelines, containing 8-39 residues covering 75% of the sequence of the influenza virus hemagglutinin HA1 polypeptide chain, induced antibodies that reacted with the HA1 protein or intact virus; and 12/12 peptides from the MuLV polymerase and 18/18 from the rabies glycoprotein induced antibodies that precipitated the respective proteins.

Antigenic epitope-bearing peptides and polypeptides of the invention are therefore useful to raise antibodies, including monoclonal antibodies, that bind specifically to a polypeptide of the invention. Thus, a high proportion of hybridomas obtained by fusion of spleen cells from donors immunized with an antigen epitope-bearing peptide generally secrete antibody reactive with the native protein. *See* Sutcliffe, et al., *supra*, p. 663. The antibodies raised by antigenic epitope-bearing peptides or polypeptides are useful to detect the mimicked protein, and antibodies to different peptides may be used for tracking the fate of various regions of a protein precursor which undergoes post-translational processing. The peptides and anti-peptide antibodies may be used in a variety of qualitative or quantitative assays for the mimicked protein, for instance in competition assays since it has been shown that even short peptides (*e.g.*, about 9 amino acids) can bind and displace the larger peptides in immunoprecipitation assays. *See, e.g.,* Wilson, et al., (1984) Cell 37:767-778. The anti-peptide antibodies of the invention also are useful for purification of the mimicked protein, for instance, by adsorption chromatography using methods known in the art.

Antigenic epitope-bearing peptides and polypeptides of the invention designed according to the above guidelines preferably contain a sequence of at least seven, more preferably at least nine and most preferably between about 10 to about 50 amino acids (i.e. any integer between 7 and 50) contained within the amino acid sequence of a polypeptide of the invention. However, peptides or polypeptides comprising a larger portion of an amino acid sequence of a polypeptide of the invention, containing about 50 to about 100 amino acids, or any length up to and including the entire amino acid sequence of a polypeptide of the invention, also are considered epitope-bearing peptides or polypeptides of the invention and also are useful for inducing antibodies that react with the mimicked protein. Preferably, the amino acid sequence of the epitope-bearing peptide is selected to provide substantial solubility in aqueous solvents (*i.e.*, the sequence includes relatively hydrophilic residues and highly hydrophobic sequences are preferably avoided); and sequences containing proline residues are particularly preferred.

Non-limiting examples of antigenic polypeptides or peptides that can be used to generate an Borrelia-specific immune response or antibodies include portions of the amino acid sequences identified in Table 1. More specifically, Table 4 discloses a list of non-limiting residues that are involved in the antigenicity of the epitope-bearing fragments of the present invention. Therefore

sequence of at least seven, more preferably at least nine and most preferably between about 10 to about 50 amino acids (i.e. any integer between 7 and 50) of a polypeptide of the present invention. That is, included in the present invention are antigenic polypeptides between the integers of 7 and 50 amino acid in length comprising one or more of the sequences of Table 4. Therefore, in most cases, the polypeptides of Table 4 make up only a portion of the antigenic polypeptide. All combinations of sequences between the integers of 7 and 50 amino acid in length comprising one or more of the sequences of Table 4 are included. The antigenic epitope-bearing fragements may be specified by either the number of contiguous amino acid residues or by specific N-terminal and C-terminal positions as described above for the polypeptide fragements of the present invention, wherein the initiation codon is residue 1. Any number of the described antigenic epitope-bearing fragements of the present invention may also be excluded from the present invention in the same manner.

The epitope-bearing peptides and polypeptides of the invention may be produced by any conventional means for making peptides or polypeptides including recombinant means using nucleic acid molecules of the invention. For instance, an epitope-bearing amino acid sequence of the present invention may be fused to a larger polypeptide which acts as a carrier during recombinant production and purification, as well as during immunization to produce anti-peptide antibodies. Epitope-bearing peptides also may be synthesized using known methods of chemical synthesis. For instance, Houghten has described a simple method for synthesis of large numbers of peptides, such as 10-20 mg of 248 different 13 residue peptides representing single amino acid variants of a segment of the HA1 polypeptide which were prepared and characterized (by ELISA-type binding studies) in less than four weeks (Houghten, R. A. Proc. Natl. Acad. Sci. USA **82:**5131-5135 (1985)). This "Simultaneous Multiple Peptide Synthesis (SMPS)" process is further described in U.S. Patent No. 4,631,211 to Houghten and coworkers (1986). In this procedure the individual resins for the solid-phase synthesis of various peptides are contained in separate solvent-permeable packets, enabling the optimal use of the many identical repetitive steps involved in solid-phase methods. A completely manual procedure allows 500-1000 or more syntheses to be conducted simultaneously (Houghten et al. (1985) Proc. Natl. Acad. Sci. 82:5131-5135 at 5134.

Epitope-bearing peptides and polypeptides of the invention are used to induce antibodies according to methods well known in the art. *See, e.g.,* Sutcliffe, et al., *supra*;; Wilson, et al., *supra*;; and Bittle, et al. (1985) J. Gen. Virol. 66**:**2347-2354. Generally, animals may be immunized with free peptide; however, anti-peptide antibody titer may be boosted by coupling of the peptide to a macromolecular carrier, such as keyhole limpet hemacyanin (KLH) or tetanus toxoid. For instance, peptides containing cysteine may be coupled to carrier using a linker such as m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), while other peptides may

carrier protein and Freund's adjuvant. Several booster injections may be needed, for instance, at intervals of about two weeks, to provide a useful titer of anti-peptide antibody which can be detected, for example, by ELISA assay using free peptide adsorbed to a solid surface. The titer of anti-peptide antibodies in serum from an immunized animal may be increased by selection of anti-peptide antibodies, for instance, by adsorption to the peptide on a solid support and elution of the selected antibodies according to methods well known in the art.

Immunogenic epitope-bearing peptides of the invention, *i.e.*, those parts of a protein that elicit an antibody response when the whole protein is the immunogen, are identified according to methods known in the art. For instance, Geysen, *et al.*, *supra*, discloses a procedure for rapid concurrent synthesis on solid supports of hundreds of peptides of sufficient purity to react in an ELISA. Interaction of synthesized peptides with antibodies is then easily detected without removing them from the support. In this manner a peptide bearing an immunogenic epitope of a desired protein may be identified routinely by one of ordinary skill in the art. For instance, the immunologically important epitope in the coat protein of foot-and-mouth disease virus was located by Geysen *et al. supra* with a resolution of seven amino acids by synthesis of an overlapping set of all 208 possible hexapeptides covering the entire 213 amino acid sequence of the protein. Then, a complete replacement set of peptides in which all 20 amino acids were substituted in turn at every position within the epitope were synthesized, and the particular amino acids conferring specificity for the reaction with antibody were determined. Thus, peptide analogs of the epitope-bearing peptides of the invention can be made routinely by this method. U.S. Patent No. 4,708,781 to Geysen (1987) further describes this method of identifying a peptide bearing an immunogenic epitope of a desired protein.

Further still, U.S. Patent No. 5,194,392, to Geysen (1990), describes a general method of detecting or determining the sequence of monomers (amino acids or other compounds) which is a topological equivalent of the epitope (*i.e.*, a "mimotope") which is complementary to a particular paratope (antigen binding site) of an antibody of interest. More generally, U.S. Patent No. 4,433,092, also to Geysen (1989), describes a method of detecting or determining a sequence of monomers which is a topographical equivalent of a ligand which is complementary to the ligand binding site of a particular receptor of interest. Similarly, U.S. Patent No. 5,480,971 to Houghten, R. A. *et al.* (1996) discloses linear C_1-C_7-alkyl peralkylated oligopeptides and sets and libraries of such peptides, as well as methods for using such oligopeptide sets and libraries for determining the sequence of a peralkylated oligopeptide that preferentially binds to an acceptor molecule of interest. Thus, non-peptide analogs of the epitope-bearing peptides of the invention also can be made routinely by these methods. The entire disclosure of each document cited in this section on "Polypeptides and Fragments" is hereby incorporated herein by reference.

As one of skill in the art will appreciate, the polypeptides of the present invention and the

chimeric proteins consisting of the first two domains of the human CD4-polypeptide and various domains of the constant regions of the heavy or light chains of mammalian immunoglobulins. (EPA 0,394,827; Traunecker et al. (1988) Nature 331:84-86. Fusion proteins that have a disulfide-linked dimeric structure due to the IgG part can also be more efficient in binding and neutralizing other molecules than a monomeric *B. burgdorferi* polypeptide or fragment thereof alone. *See* Fountoulakis et al. (1995) J. Biochem. 270:3958-3964. Nucleic acids encoding the above epitopes of *B. burgdorferi* polypeptides can also be recombined with a gene of interest as an epitope tag to aid in detection and purification of the expressed polypeptide.

Antibodies

B. burgdorferi protein-specific antibodies for use in the present invention can be raised against the intact *B. burgdorferi* protein or an antigenic polypeptide fragment thereof, which may be presented together with a carrier protein, such as an albumin, to an animal system (such as rabbit or mouse) or, if it is long enough (at least about 25 amino acids), without a carrier.

As used herein, the term "antibody" (Ab) or "monoclonal antibody" (Mab) is meant to include intact molecules, single chain whole antibodies, and antibody fragments. Antibody fragments of the present invention include Fab and F(ab')2 and other fragments including single-chain Fvs (scFv) and disulfide-linked Fvs (sdFv). Also included in the present invention are chimeric and humanized monoclonal antibodies and polyclonal antibodies specific for the polypeptides of the present invention. The antibodies of the present invention may be prepared by any of a variety of methods. For example, cells expressing a polypeptide of the present invention or an antigenic fragment thereof can be administered to an animal in order to induce the production of sera containing polyclonal antibodies. For example, a preparation of *B. burgdorferi* polypeptide or fragment thereof is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

In a preferred method, the antibodies of the present invention are monoclonal antibodies or binding fragments thereof. Such monoclonal antibodies can be prepared using hybridoma technology. *See, e.g.,* Harlow et al., ANTIBODIES: A LABORATORY MANUAL, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988); Hammerling, et al., in: MONOCLONAL ANTIBODIES AND T-CELL HYBRIDOMAS 563-681 (Elsevier, N.Y., 1981). Fab and F(ab')2 fragments may be produced by proteolytic cleavage, using enzymes such as papain (to produce Fab fragments) or pepsin (to produce F(ab')2 fragments). Alternatively, *B. burgdorferi* polypeptide-binding fragments, chimeric, and humanized antibodies can be produced through the application of recombinant DNA technology or through synthetic chemistry using methods known in the art.

Alternatively, additional antibodies capable of binding to the polypeptide antigen of the present invention may be produced in a two-step procedure through the use of

therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, *B. burgdorferi* polypeptide-specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the *B. burgdorferi* polypeptide-specific antibody can be blocked by the *B. burgdorferi* polypeptide antigen. Such antibodies comprise anti-idiotypic antibodies to the *B. burgdorferi* polypeptide-specific antibody and can be used to immunize an animal to induce formation of further *B. burgdorferi* polypeptide-specific antibodies.

Antibodies and fragements thereof of the present invention may be described by the portion of a polypeptide of the present invention recognized or specifically bound by the antibody. Antibody binding fragements of a polypeptide of the present invention may be described or specified in the same manner as for polypeptide fragements discussed above., i.e, by N-terminal and C-terminal positions or by size in contiguous amino acid residues. Any number of antibody binding fragments, of a polypeptide of the present invention, specified by N-terminal and C-terminal positions or by size in amino acid residues, as described above, may also be excluded from the present invention. Therefore, the present invention includes antibodies the specifically bind a particuarlly discribed fragement of a polypeptide of the present invention and allows for the exclusion of the same.

Antibodies and fragements thereof of the present invention may also be described or specified in terms of their cross-reactivity. Antibodies and fragements that do not bind polypeptides of any other species of *Borrelia* other than *B. burgdorferi* are included in the present invention. Likewise, antibodies and fragements that bind only species of *Borrelia*, i.e. antibodies and fragements that do not bind bacteria from any genus other than *Borrelia*, are included in the present invention.

Diagnostic Assays

The present invention further relates to methods for assaying *staphylococcal* infection in an animal by detecting the expression of genes encoding *staphylococcal* polypeptides of the present invention. The methods comprise analyzing tissue or body fluid from the animal for *Borrelia*-specific antibodies, nucleic acids, or proteins. Analysis of nucleic acid specific to *Borrelia* is assayed by PCR or hybridization techniques using nucleic acid sequences of the present invention as either hybridization probes or primers. *See, e.g.,* Sambrook et al. Molecular cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, 2nd ed., 1989, page 54 reference); Eremeeva et al. (1994) J. Clin. Microbiol. 32:803-810 (describing differentiation among spotted fever group *Rickettsiae* species by analysis of restriction fragment length polymorphism of PCR-amplified DNA) and Chen et al. 1994 J. Clin. Microbiol. 32:589-595 (detecting *B. burgdorferi* nucleic acids *via* PCR).

Where diagnosis of a disease state related to infection with *Borrelia* has already been made, the present invention is useful for monitoring progression or regression of the disease state

whereby patients exhibiting enhanced *Borrelia* gene expression will experience a worse clinical outcome relative to patients expressing these gene(s) at a lower level.

By "biological sample" is intended any biological sample obtained from an animal, cell line, tissue culture, or other source which contains *Borrelia* polypeptide, mRNA, or DNA. Biological samples include body fluids (such as saliva, blood, plasma, urine, mucus, synovial fluid, etc.) tissues (such as muscle, skin, and cartilage) and any other biological source suspected of containing *Borrelia* polypeptides or nucleic acids. Methods for obtaining biological samples such as tissue are well known in the art.

The present invention is useful for detecting diseases related to *Borrelia* infections in animals. Preferred animals include monkeys, apes, cats, dogs, birds, cows, pigs, mice, horses, rabbits and humans. Particularly preferred are humans.

Total RNA can be isolated from a biological sample using any suitable technique such as the single-step guanidinium-thiocyanate-phenol-chloroform method described in Chomczynski et al. (1987) Anal. Biochem. 162:156-159. mRNA encoding *Borrelia* polypeptides having sufficient homology to the nucleic acid sequences identified in Table 1 to allow for hybridization between complementary sequences are then assayed using any appropriate method. These include Northern blot analysis, S1 nuclease mapping, the polymerase chain reaction (PCR), reverse transcription in combination with the polymerase chain reaction (RT-PCR), and reverse transcription in combination with the ligase chain reaction (RT-LCR).

Northern blot analysis can be performed as described in Harada et al. (1990) Cell 63:303-312. Briefly, total RNA is prepared from a biological sample as described above. For the Northern blot, the RNA is denatured in an appropriate buffer (such as glyoxal/dimethyl sulfoxide/sodium phosphate buffer), subjected to agarose gel electrophoresis, and transferred onto a nitrocellulose filter. After the RNAs have been linked to the filter by a UV linker, the filter is prehybridized in a solution containing formamide, SSC, Denhardt's solution, denatured salmon sperm, SDS, and sodium phosphate buffer. A *B. burgdorferi* polynucleotide sequence shown in Table 1 labeled according to any appropriate method (such as the ^{32}P-multiprimed DNA labeling system (Amersham)) is used as probe. After hybridization overnight, the filter is washed and exposed to x-ray film. DNA for use as probe according to the present invention is described in the sections above and will preferably at least 15 nucleotides in length.

S1 mapping can be performed as described in Fujita et al. (1987) Cell 49:357-367. To prepare probe DNA for use in S1 mapping, the sense strand of an above-described *B. burgdorferi* DNA sequence of the present invention is used as a template to synthesize labeled antisense DNA. The antisense DNA can then be digested using an appropriate restriction endonuclease to generate further DNA probes of a desired length. Such antisense probes are useful for visualizing protected bands corresponding to the target mRNA (*i.e.* mRNA encoding *Borrelia* polypeptides)

concentration of the target mRNA. Briefly, this method involves adding total RNA isolated from a biological sample in a reaction mixture containing a RT primer and appropriate buffer. After incubating for primer annealing, the mixture can be supplemented with a RT buffer, dNTPs, DTT, RNase inhibitor and reverse transcriptase. After incubation to achieve reverse transcription of the RNA, the RT products are then subject to PCR using labeled primers. Alternatively, rather than labeling the primers, a labeled dNTP can be included in the PCR reaction mixture. PCR amplification can be performed in a DNA thermal cycler according to conventional techniques. After a suitable number of rounds to achieve amplification, the PCR reaction mixture is electrophoresed on a polyacrylamide gel. After drying the gel, the radioactivity of the appropriate bands (corresponding to the mRNA encoding the *Borrelia* polypeptides of the present invention) are quantified using an imaging analyzer. RT and PCR reaction ingredients and conditions, reagent and gel concentrations, and labeling methods are well known in the art. Variations on the RT-PCR method will be apparent to the skilled artisan. Other PCR methods that can detect the nucleic acid of the present invention can be found in PCR PRIMER: A LABORATORY MANUAL (C.W. Dieffenbach et al. eds., Cold Spring Harbor Lab Press, 1995).

The polynucleotides of the present invention, including both DNA and RNA, may be used to detect polynucleotides of the present invention or Borrelia species including *B. burgdorferi* using bio chip technology. The present invention includes both high density chip arrays (>1000 oligonucleotides per cm^2) and low density chip arrays (<1000 oligonucleotides per cm^2). Bio chips comprising arrays of polynucleotides of the present invention may be used to detect Borrelia species, including *B. burgdorferi*, in biological and environmental samples and to diagnose an animal, including humans, with an *B. burgdorferi* or other Borrelia infection. The bio chips of the present invention may comprise polynucleotide sequences of other pathogens including bacteria, viral, parasitic, and fungal polynucleotide sequences, in addition to the polynucleotide sequences of the present invention, for use in rapid diffenertial pathogenic detection and diagnosis. The bio chips can also be used to monitor an *B. burgdorferi* or other Borrelia infections and to monitor the genetic changes (deletions, insertions, mismatches, etc.) in response to drug therapy in the clinic and drug development in the laboratory. The bio chip technology comprising arrays of polynucleotides of the present invention may also be used to simultaneously monitor the expression of a multiplicity of genes, including those of the present invention. The polynucleotides used to comprise a selected array may be specified in the same manner as for the fragements, i.e, by their 5' and 3' positions or length in contigious base pairs and include from. Methods and particular uses of the polynucleotides of the present invention to detect Borrelia species, including *B. burgdorferi*, using bio chip technology include those known in the art and those of: U.S. Patent Nos. 5510270, 5545531, 5445934, 5677195, 5532128, 5556752,

Biosensors using the polynucleotides of the present invention may also be used to detect particular polynucleotides of the present invention. Biosensors using the polynucleotides of the present invention may also be used to monitor the genetic changes (deletions, insertions, mismatches, etc.) in response to drug therapy in the clinic and drug development in the laboratory. Methods and particular uses of the polynucleotides of the present invention to detect Borrelia species, including *B. burgdorferi*, using biosenors include those known in the art and those of: U.S. Patent Nos 5721102, 5658732, 5631170, and World Patent Nos. WO97/35011, WO/9720203, each incorporated herein in their entireties.

Thus, the present invention includes both bio chips and biosensors comprising polynucleotides of the present invention and methods of their use.

Assaying *Borrelia* polypeptide levels in a biological sample can occur using any art-known method, such as antibody-based techniques. For example, *Borrelia* polypeptide expression in tissues can be studied with classical immunohistological methods. In these, the specific recognition is provided by the primary antibody (polyclonal or monoclonal) but the secondary detection system can utilize fluorescent, enzyme, or other conjugated secondary antibodies. As a result, an immunohistological staining of tissue section for pathological examination is obtained. Tissues can also be extracted, *e.g.*, with urea and neutral detergent, for the liberation of *Borrelia* polypeptides for Western-blot or dot/slot assay. *See, e.g.*, Jalkanen, M. et al. (1985) J. Cell. Biol. 101:976-985; Jalkanen, M. et al. (1987) J. Cell . Biol. 105:3087-3096. In this technique, which is based on the use of cationic solid phases, quantitation of a *Borrelia* polypeptide can be accomplished using an isolated *Borrelia* polypeptide as a standard. This technique can also be applied to body fluids.

Other antibody-based methods useful for detecting *Borrelia* polypeptide gene expression include immunoassays, such as the ELISA and the radioimmunoassay (RIA). For example, a *Borrelia* polypeptide-specific monoclonal antibodies can be used both as an immunoabsorbent and as an enzyme-labeled probe to detect and quantify a *Borrelia* polypeptide. The amount of a *Borrelia* polypeptide present in the sample can be calculated by reference to the amount present in a standard preparation using a linear regression computer algorithm. Such an ELISA is described in Iacobelli et al. (1988) Breast Cancer Research and Treatment 11:19-30. In another ELISA assay, two distinct specific monoclonal antibodies can be used to detect *Borrelia* polypeptides in a body fluid. In this assay, one of the antibodies is used as the immunoabsorbent and the other as the enzyme-labeled probe.

The above techniques may be conducted essentially as a "one-step" or "two-step" assay. The "one-step" assay involves contacting the *Borrelia* polypeptide with immobilized antibody and, without washing, contacting the mixture with the labeled antibody. The "two-step" assay involves washing before contacting the mixture with the labeled antibody. Other conventional

and other immunological methods included in the present invention can also be found in Harlow et al., ANTIBODIES: A LABORATORY MANUAL, (Cold Spring Harbor Laboratory Press, 2nd ed. 1988).

Suitable enzyme labels include, for example, those from the oxidase group, which catalyze the production of hydrogen peroxide by reacting with substrate. Glucose oxidase is particularly preferred as it has good stability and its substrate (glucose) is readily available. Activity of an oxidase label may be assayed by measuring the concentration of hydrogen peroxide formed by the enzyme-labeled antibody/substrate reaction. Besides enzymes, other suitable labels include radioisotopes, such as iodine (^{125}I, ^{121}I), carbon (^{14}C), sulphur (^{35}S), tritium (^{3}H), indium (^{112}In), and technetium (^{99m}Tc), and fluorescent labels, such as fluorescein and rhodamine, and biotin.

Further suitable labels for the *Borrelia* polypeptide-specific antibodies of the present invention are provided below. Examples of suitable enzyme labels include malate dehydrogenase, Borrelia nuclease, delta-5-steroid isomerase, yeast-alcohol dehydrogenase, alpha-glycerol phosphate dehydrogenase, triose phosphate isomerase, peroxidase, alkaline phosphatase, asparaginase, glucose oxidase, beta-galactosidase, ribonuclease, urease, catalase, glucose-6-phosphate dehydrogenase, glucoamylase, and acetylcholine esterase.

Examples of suitable radioisotopic labels include ^{3}H, ^{111}In, ^{125}I, ^{131}I, ^{32}P, ^{35}S, ^{14}C, ^{51}Cr, ^{57}To, ^{58}Co, ^{59}Fe, ^{75}Se, ^{152}Eu, ^{90}Y, ^{67}Cu, ^{217}Ci, ^{211}At, ^{212}Pb, ^{47}Sc, ^{109}Pd, etc. ^{111}In is a preferred isotope where *in vivo* imaging is used since its avoids the problem of dehalogenation of the ^{125}I or ^{131}I-labeled monoclonal antibody by the liver. In addition, this radionucleotide has a more favorable gamma emission energy for imaging. *See, e.g.,* Perkins et al. (1985) Eur. J. Nucl. Med. 10:296-301; Carasquillo et al. (1987) J. Nucl. Med. 28:281-287. For example, ^{111}In coupled to monoclonal antibodies with 1-(P-isothiocyanatobenzyl)-DPTA has shown little uptake in non-tumors tissues, particularly the liver, and therefore enhances specificity of tumor localization. See, Esteban et al. (1987) J. Nucl. Med. 28:861-870.

Examples of suitable non-radioactive isotopic labels include ^{157}Gd, ^{55}Mn, ^{162}Dy, ^{52}Tr, and ^{56}Fe.

Examples of suitable fluorescent labels include an ^{152}Eu label, a fluorescein label, an isothiocyanate label, a rhodamine label, a phycoerythrin label, a phycocyanin label, an allophycocyanin label, an o-phthaldehyde label, and a fluorescamine label.

Examples of suitable toxin labels include, *Pseudomonas* toxin, diphtheria toxin, ricin, and cholera toxin.

Examples of chemiluminescent labels include a luminal label, an isoluminal label, an aromatic acridinium ester label, an imidazole label, an acridinium salt label, an oxalate ester label, a luciferin label, a luciferase label, and an aequorin label.

Examples of nuclear magnetic resonance contrasting agents include heavy metal nuclei such as Gd, Mn, and iron.

Typical techniques for binding the above-described labels to antibodies are provided by

Kennedy et al. (1976) Clin. Chim. Acta 70:1-31, and Schurs et al. (1977) Clin. Chim. Acta 81:1-40. Coupling techniques mentioned in the latter are the glutaraldehyde method, the periodate method, the dimaleimide method, the m-maleimidobenzyl-N-hydroxy-succinimide ester method, all of which methods are incorporated by reference herein.

In a related aspect, the invention includes a diagnostic kit for use in screening serum containing antibodies specific against *B. burgdorferi* infection. Such a kit may include an isolated *B. burgdorferi* antigen comprising an epitope which is specifically immunoreactive with at least one anti-*B. burgdorferi* antibody. Such a kit also includes means for detecting the binding of said antibody to the antigen. In specific embodiments, the kit may include a recombinantly produced or chemically synthesized peptide or polypeptide antigen. The peptide or polypeptide antigen may be attached to a solid support.

In a more specific embodiment, the detecting means of the above-described kit includes a solid support to which said peptide or polypeptide antigen is attached. Such a kit may also include a non-attached reporter-labeled anti-human antibody. In this embodiment, binding of the antibody to the *B. burgdorferi* antigen can be detected by binding of the reporter labeled antibody to the anti-*B. burgdorferi* polypeptide antibody.

In a related aspect, the invention includes a method of detecting *B. burgdorferi* infection in a subject. This detection method includes reacting a body fluid, preferably serum, from the subject with an isolated *B. burgdorferi* antigen, and examining the antigen for the presence of bound antibody. In a specific embodiment, the method includes a polypeptide antigen attached to a solid support, and serum is reacted with the support. Subsequently, the support is reacted with a reporter-labeled anti-human antibody. The support is then examined for the presence of reporter-labeled antibody.

The solid surface reagent employed in the above assays and kits is prepared by known techniques for attaching protein material to solid support material, such as polymeric beads, dip sticks, 96-well plates or filter material. These attachment methods generally include non-specific adsorption of the protein to the support or covalent attachment of the protein , typically through a free amine group, to a chemically reactive group on the solid support, such as an activated carboxyl, hydroxyl, or aldehyde group. Alternatively, streptavidin coated plates can be used in conjunction with biotinylated antigen(s).

The polypeptides and antibodies of the present invention, including fragments thereof, may be used to detect Borrelia species including *B. burgdorferi* using bio chip and biosensor technology. Bio chip and biosensors of the present invention may comprise the polypeptides of the present invention to detect antibodies, which specifically recognize Borrelia species, including *B. burgdorferi*. Bio chip and biosensors of the present invention may also comprise antibodies which specifically recognize the polypeptides of the present invention to detect Borrelia species, including *B. burgdorferi* or specific polypeptides of the present invention. Bio chip

diagnose an animal, including humans, with an *B. burgdorferi* or other Borrelia infection. Thus, the present invention includes both bio chips and biosensors comprising polypeptides or antibodies of the present invention and methods of their use.

The bio chips of the present invention may further comprise polypeptide sequences of other pathogens including bacteria, viral, parasitic, and fungal polypeptide sequences, in addition to the polypeptide sequences of the present invention, for use in rapid diffenertial pathogenic detection and diagnosis. The bio chips of the present invention may further comprise antibodies or fragements thereof specific for other pathogens including bacteria, viral, parasitic, and fungal polypeptide sequences, in addition to the antibodies or fragements thereof of the present invention, for use in rapid diffenertial pathogenic detection and diagnosis. The bio chips and biosensors of the present invention may also be used to monitor an *B. burgdorferi* or other Borrelia infection and to monitor the genetic changes (amio acid deletions, insertions, substitutions, etc.) in response to drug therapy in the clinic and drug development in the laboratory. The bio chip and biosensors comprising polypeptides or antibodies of the present invention may also be used to simultaneously monitor the expression of a multiplicity of polypeptides, including those of the present invention. The polypeptides used to comprise a bio chip or biosensor of the present invention may be specified in the same manner as for the fragements, i.e, by their N-terminal and C-terminal positions or length in contigious amino acid residue. Methods and particular uses of the polypeptides and antibodies of the present invention to detect Borrelia species, including *B. burgdorferi*, or specific polypeptides using bio chip and biosensor technology include those known in the art, those of the U.S. Patent Nos. and World Patent Nos. listed above for bio chips and biosensors using polynucleotides of the present invention, and those of: U.S. Patent Nos. 5658732, 5135852, 5567301, 5677196, 5690894 and World Patent Nos. WO9729366, WO9612957, each incorporated herein in their entireties.

Treatment:

Agonists and Antagonists - Assays and Molecules

The invention also provides a method of screening compounds to identify those which enhance or block the biological activity of the *B. burgdorferi* polypeptides of the present invention. The present invention further provides where the compounds kill or slow the growth of *B. burgdorferi*. The ability of *B. burgdorferi* antagonists, including *B. burgdorferi* ligands, to prophylactically or therapeutically block antibiotic resistance may be easily tested by the skilled artisan. *See, e.g.,* Straden et al. (1997) J Bacteriol. 179(1):9-16.

An agonist is a compound which increases the natural biological function or which functions in a manner similar to the polypeptides of the present invention, while antagonists decrease or eliminate such functions. Potential antagonists include small organic molecules, peptides, polypeptides, and antibodies that bind to a polypeptide of the invention and thereby inhibit or extinguish its activity.

The antagonists may be employed for instance to inhibit peptidoglycan cross bridge

formation. Antibodies against *B. burgdorferi* may be employed to bind to and inhibit *B. burgdorferi* activity to treat antibiotic resistance. Any of the above antagonists may be employed in a composition with a pharmaceutically acceptable carrier.

Vaccines

The present invention also provides vaccines comprising one or more polypeptides of the present invention. Heterogeneity in the composition of a vaccine may be provided by combining *B. burgdorferi* polypeptides of the present invention. Multi-component vaccines of this type are desirable because they are likely to be more effective in eliciting protective immune responses against multiple species and strains of the *Borrelia* genus than single polypeptide vaccines. Thus, as discussed in detail below, a multi-component vaccine of the present invention may contain one or more, preferably 2 to about 20, more preferably 2 to about 15, and most preferably 3 to about 8, of the *B. burgdorferi* polypeptides shown in Table 1, or fragments thereof.

Multi-component vaccines are known in the art to elicit antibody production to numerous immunogenic components. Decker, M. and Edwards, K., *J. Infect. Dis. 174*:S270-275 (1996). In addition, a hepatitis B, diphtheria, tetanus, pertussis tetravalent vaccine has recently been demonstrated to elicit protective levels of antibodies in human infants against all four pathogenic agents. Aristegui, J. *et al., Vaccine 15*:7-9 (1997).

The present invention thus also includes multi-component vaccines. These vaccines comprise more than one polypeptide, immunogen or antigen. An example of such a multi-component vaccine would be a vaccine comprising more than one of the *B. burgdorferi* polypeptides shown in Table 1. A second example is a vaccine comprising one or more, for example 2 to 10, of the *B. burgdorferi* polypeptides shown in Table 1 and one or more, for example 2 to 10, additional polypeptides of either borrelial or non-borrelial origin. Thus, a multi-component vaccine which confers protective immunity to both a borrelial infection and infection by another pathogenic agent is also within the scope of the invention.

As indicated above, the vaccines of the present invention are expected to elicit a protective immune response against infections caused by species and strains of *Borrelia* other than *B. burgdorferi* sensu stricto isolate B31 (ATCC Accession No. 35210). Immunizations using decorin-binding protein and OspA derived from one strain of *B. burgdorferi* has been shown to elicit the production of antiserum which confers passive immunity against other strains of *B. burgdorferi*. Cassatt, D. *et al., Protection of Borrelia burgdorferi Infection by Antibodies to Decorin-binding Protein*, in VACCINES97, Cold Spring Harbor Press (1997), pages 191-195. Further, the inventors have found using an *in vitro* assay that antiserum produced in response to *B. burgdorferi* decorin-binding protein will kill several species of *Borrelia*. The amino acid sequences of decorin-binding protein expressed by different strains of *B. burgdorferi* are believed to diverge by as much as 25%. Thus, antisera elicited against decorin-binding proteins confers passive immunity against *Borrelia* expressing proteins having only 75% or less amino

Further within the scope of the invention are whole cell and whole viral vaccines. Such vaccines may be produced recombinantly and involve the expression of one or more of the *B. burgdorferi* polypeptides shown in Table 1. For example, the *B. burgdorferi* polypeptides of the present invention may be either secreted or localized intracellular, on the cell surface, or in the periplasmic space. Further, when a recombinant virus is used, the *B. burgdorferi* polypeptides of the present invention may, for example, be localized in the viral envelope, on the surface of the capsid, or internally within the capsid. Whole cells vaccines which employ cells expressing heterologous proteins are known in the art. *See, e.g.*, Robinson, K. *et al., Nature Biotech. 15*:653-657 (1997); Sirard, J. *et al., Infect. Immun. 65*:2029-2033 (1997); Chabalgoity, J. *et al., Infect. Immun. 65*:2402-2412 (1997). These cells may be administered live or may be killed prior to administration. Chabalgoity, J. *et al., supra*, for example, report the successful use in mice of a live attenuated *Salmonella* vaccine strain which expresses a portion of a platyhelminth fatty acid-binding protein as a fusion protein on its cells surface.

A multi-component vaccine can also be prepared using techniques known in the art by combining one or more *B. burgdorferi* polypeptides of the present invention, or fragments thereof, with additional non-borrelial components (*e.g.*, diphtheria toxin or tetanus toxin, and/or other compounds known to elicit an immune response). Such vaccines are useful for eliciting protective immune responses to both members of the *Borrelia* genus and non-borrelial pathogenic agents.

The vaccines of the present invention also include DNA vaccines. DNA vaccines are currently being developed for a number of infectious diseases. Boyer, J *et al., Nat. Med. 3*:526-532 (1997); reviewed in Spier, R., *Vaccine 14*:1285-1288 (1996). Such DNA vaccines contain a nucleotide sequence encoding one or more *B. burgdorferi* polypeptides of the present invention oriented in a manner that allows for expression of the subject polypeptide. The direct administration of plasmid DNA encoding OspA has been shown to elicit protective immunity in mice against borrelial challenge. Luke, C. *et al., J. Infect. Dis. 175*:91-97 (1997).

The present invention also relates to the administration of a vaccine which is co-administered with a molecule capable of modulating immune responses. Kim, J. *et al., Nature Biotech. 15*:641-646 (1997), for example, report the enhancement of immune responses produced by DNA immunizations when DNA sequences encoding molecules which stimulate the immune response are co-administered. In a similar fashion, the vaccines of the present invention may be co-administered with either nucleic acids encoding immune modulators or the immune modulators themselves. These immune modulators include granulocyte macrophage colony stimulating factor (GM-CSF) and CD86.

The vaccines of the present invention may be used to confer resistance to borrelial infection by either passive or active immunization. When the vaccines of the present invention are used to confer resistance to borrelial infection through active immunization, a vaccine of the present invention is administered to an animal to elicit a protective immune response which either

confer resistance to borrelial infection through passive immunization, the vaccine is provided to a host animal (*e.g.*, human, dog, or mouse), and the antisera elicited by this antisera is recovered and directly provided to a recipient suspected of having an infection caused by a member of the *Borrelia* genus.

The ability to label antibodies, or fragments of antibodies, with toxin molecules provides an additional method for treating borrelial infections when passive immunization is conducted. In this embodiment, antibodies, or fragments of antibodies, capable of recognizing the *B. burgdorferi* polypeptides disclosed herein, or fragments thereof, as well as other *Borrelia* proteins, are labeled with toxin molecules prior to their administration to the patient. When such toxin derivatized antibodies bind to *Borrelia* cells, toxin moieties will be localized to these cells and will cause their death.

The present invention thus concerns and provides a means for preventing or attenuating a borrelial infection resulting from organisms which have antigens that are recognized and bound by antisera produced in response to the polypeptides of the present invention. As used herein, a vaccine is said to prevent or attenuate a disease if its administration to an animal results either in the total or partial attenuation (*i.e.*, suppression) of a symptom or condition of the disease, or in the total or partial immunity of the animal to the disease.

The administration of the vaccine (or the antisera which it elicits) may be for either a "prophylactic" or "therapeutic" purpose. When provided prophylactically, the compound(s) are provided in advance of any symptoms of borrelial infection. The prophylactic administration of the compound(s) serves to prevent or attenuate any subsequent infection. When provided therapeutically, the compound(s) is provided upon or after the detection of symptoms which indicate that an animal may be infected with a member of the *Borrelia* genus. The therapeutic administration of the compound(s) serves to attenuate any actual infection. Thus, the *B. burgdorferi* polypeptides, and fragments thereof, of the present invention may be provided either prior to the onset of infection (so as to prevent or attenuate an anticipated infection) or after the initiation of an actual infection.

The polypeptides of the invention, whether encoding a portion of a native protein or a functional derivative thereof, may be administered in pure form or may be coupled to a macromolecular carrier. Example of such carriers are proteins and carbohydrates. Suitable proteins which may act as macromolecular carrier for enhancing the immunogenicity of the polypeptides of the present invention include keyhole limpet hemacyanin (KLH) tetanus toxoid, pertussis toxin, bovine serum albumin, and ovalbumin. Methods for coupling the polypeptides of the present invention to such macromolecular carriers are disclosed in Harlow *et al., Antibodies: A Laboratory Manual, 2nd Ed.;* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988), the entire disclosure of which is incorporated by reference herein.

A composition is said to be "pharmacologically acceptable" if its administration can be

is physiologically significant. An agent is physiologically significant if its presence results in a detectable change in the physiology of a recipient patient.

While in all instances the vaccine of the present invention is administered as a pharmacologically acceptable compound, one skilled in the art would recognize that the composition of a pharmacologically acceptable compound varies with the animal to which it is administered. For example, a vaccine intended for human use will generally not be co-administered with Freund's adjuvant. Further, the level of purity of the *B. burgdorferi* polypeptides of the present invention will normally be higher when administered to a human than when administered to a non-human animal.

As would be understood by one of ordinary skill in the art, when the vaccine of the present invention is provided to an animal, it may be in a composition which may contain salts, buffers, adjuvants, or other substances which are desirable for improving the efficacy of the composition. Adjuvants are substances that can be used to specifically augment a specific immune response. These substances generally perform two functions: (1) they protect the antigen(s) from being rapidly catabolized after administration and (2) they nonspecifically stimulate immune responses.

Normally, the adjuvant and the composition are mixed prior to presentation to the immune system, or presented separately, but into the same site of the animal being immunized. Adjuvants can be loosely divided into several groups based upon their composition. These groups include oil adjuvants (for example, Freund's complete and incomplete), mineral salts (for example, $AlK(SO_4)_2$, $AlNa(SO_4)_2$, $AlNH_4(SO_4)$, silica, kaolin, and carbon), polynucleotides (for example, poly IC and poly AU acids), and certain natural substances (for example, wax D from *Mycobacterium tuberculosis*, as well as substances found in *Corynebacterium parvum*, or *Bordetella pertussis*, and members of the genus *Brucella*. Other substances useful as adjuvants are the saponins such as, for example, Quil A. (Superfos A/S, Denmark). Preferred adjuvants for use in the present invention include aluminum salts, such as $AlK(SO_4)_2$, $AlNa(SO_4)_2$, and $AlNH_4(SO_4)$. Examples of materials suitable for use in vaccine compositions are provided in *Remington's Pharmaceutical Sciences* (Osol, A, Ed, Mack Publishing Co, Easton, PA, pp. 1324-1341 (1980), which reference is incorporated herein by reference).

The therapeutic compositions of the present invention can be administered parenterally by injection, rapid infusion, nasopharyngeal absorption (intranasopharangeally), dermoabsorption, or orally. The compositions may alternatively be administered intramuscularly, or intravenously. Compositions for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Carriers or occlusive dressings can be used to increase skin permeability and enhance antigen absorption. Liquid dosage forms for oral administration may generally comprise a

commonly used in the art, such as purified water. Besides the inert diluents, such compositions can also include adjuvants, wetting agents, emulsifying and suspending agents, or sweetening, flavoring, or perfuming agents.

Therapeutic compositions of the present invention can also be administered in encapsulated form. For example, intranasal immunization of mice against *Bordetella pertussis* infection using vaccines encapsulated in biodegradable microsphere composed of poly(DL-lactide-co-glycolide) has been shown to stimulate protective immune responses. Shahin, R. *et al., Infect. Immun. 63*:1195-1200 (1995). Similarly, orally administered encapsulated *Salmonella typhimurium* antigens have also been shown to elicit protective immunity in mice. Allaoui-Attarki, K. *et al., Infect. Immun. 65*:853-857 (1997). Encapsulated vaccines of the present invention can be administered by a variety of routes including those involving contacting the vaccine with mucous membranes (*e.g.*, intranasally, intracolonicly, intraduodenally).

Many different techniques exist for the timing of the immunizations when a multiple administration regimen is utilized. It is possible to use the compositions of the invention more than once to increase the levels and diversities of expression of the immunoglobulin repertoire expressed by the immunized animal. Typically, if multiple immunizations are given, they will be given one to two months apart.

According to the present invention, an "effective amount" of a therapeutic composition is one which is sufficient to achieve a desired biological effect. Generally, the dosage needed to provide an effective amount of the composition will vary depending upon such factors as the animal's or human's age, condition, sex, and extent of disease, if any, and other variables which can be adjusted by one of ordinary skill in the art.

The antigenic preparations of the invention can be administered by either single or multiple dosages of an effective amount. Effective amounts of the compositions of the invention can vary from 0.01-1,000 µg/ml per dose, more preferably 0.1-500 µg/ml per dose, and most preferably 10-300 µg/ml per dose.

Having now generally described the invention, the same will be more readily understood through reference to the following example which is provided by way of illustration, and is not intended to be limiting of the present invention, unless specified.

Examples

1. Preparation of PCR Primers and Amplification of DNA

Various fragments of the *Borrelia burgdorferi* genome, such as those of Table 1, can be used, in accordance with the present invention, to prepare PCR primers for a variety of uses. The PCR primers are preferably at least 15 bases, and more preferably at least 18 bases in length. When selecting a primer sequence, it is preferred that the primer pairs have approximately the same G/C ratio, so that melting temperatures are approximately the same. The PCR primers and

amplified DNA of this Example find use in the Examples that follow.

2. Isolation of a Selected DNA Clone From B. burgdorferi

Three approaches are used to isolate a *B. burgdorferi* clone comprising a polynucleotide of the present invention from any *B. burgdorferi* genomic DNA library. The *B. burgdorferi* strain B31PU has been deposited as a convienent source for obtaining a *B. burgdorferi* strain although a wide varity of strains *B. burgdorferi* strains can be used which are known in the art.

B. burgdorferi genomic DNA is prepared using the following method. A 20ml overnight bacterial culture grown in a rich medium (e.g., Trypticase Soy Broth, Brain Heart Infusion broth or Super broth), pelleted, ished two times with TES (3OmM Tris-pH 8.0, 25mM EDTA, 50mM NaCl), and resuspended in 5ml high salt TES (2.5M NaCl). Lysostaphin is added to final concentration of approx 50ug/ml and the mixture is rotated slowly 1 hour at 37C to make protoplast cells. The solution is then placed in incubator (or place in a shaking water bath) and warmed to 55C. Five hundred micro liter of 20% sarcosyl in TES (final concentration 2%) is then added to lyse the cells. Next, guanidine HCl is added to a final concentration of 7M (3.69g in 5.5 ml). The mixture is swirled slowly at 55C for 60-90 min (solution should clear). A CsCl gradient is then set up in SW41 ultra clear tubes using 2.0ml 5.7M CsCl and overlaying with 2.85M CsCl. The gradient is carefully overlayed with the DNA-containing GuHCl solution. The gradient is spun at 30,000 rpm, 20C for 24 hr and the lower DNA band is collected. The volume is increased to 5 ml with TE buffer. The DNA is then treated with protease K (10 ug/ml) overnight at 37 C, and precipitated with ethanol. The precipitated DNA is resuspended in a desired buffer.

In the first method, a plasmid is directly isolated by screening a plasmid *B. burgdorferi* genomic DNA library using a polynucleotide probe corresponding to a polynucleotide of the present invention. Particularly, a specific polynucleotide with 30-40 nucleotides is synthesized using an Applied Biosystems DNA synthesizer according to the sequence reported. The oligonucleotide is labeled, for instance, with ^{32}P-γ-ATP using T4 polynucleotide kinase and purified according to routine methods. (*See, e.g.*, Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring, NY (1982).) The library is transformed into a suitable host, as indicated above (such as XL-1 Blue (Stratagene)) using techniques known to those of skill in the art. *See, e.g.*, Sambrook et al. MOLECULAR CLONING: A LABORATORY MANUAL (Cold Spring Harbor, N.Y. 2nd ed. 1989); Ausubel et al., CURRENT PROTOCALS IN MOLECULAR BIOLOGY (John Wiley and Sons, N.Y. 1989). The transformants are plated on 1.5% agar plates (containing the appropriate selection agent, e.g., ampicillin) to a density of about 150 transformants (colonies) per plate. These plates are screened using Nylon membranes according to routine methods for bacterial colony screening. *See, e.g.*, Sambrook et al. MOLECULAR CLONING: A LABORATORY MANUAL (Cold Spring Harbor, N.Y. 2nd ed. 1989); Ausubel et al., CURRENT PROTOCALS IN

MOLECULAR BIOLOGY (John Wiley and Sons, N.Y. 1989) or other techniques known to those of skill in the art.

Alternatively, two primers of 15-25 nucleotides derived from the 5' and 3' ends of a polynucleotide of Table 1 are synthesized and used to amplify the desired DNA by PCR using a *B. burgdorferi* genomic DNA prep as a template. PCR is carried out under routine conditions, for instance, in 25 µl of reaction mixture with 0.5 ug of the above DNA template. A convenient reaction mixture is 1.5-5 mM $MgCl_2$, 0.01% (w/v) gelatin, 20 µM each of dATP, dCTP, dGTP, dTTP, 25 pmol of each primer and 0.25 Unit of Taq polymerase. Thirty five cycles of PCR (denaturation at 94°C for 1 min; annealing at 55°C for 1 min; elongation at 72°C for 1 min) are performed with a Perkin-Elmer Cetus automated thermal cycler. The amplified product is analyzed by agarose gel electrophoresis and the DNA band with expected molecular weight is excised and purified. The PCR product is verified to be the selected sequence by subcloning and sequencing the DNA product.

Finally, overlapping oligos of the DNA sequences of Table 1 can be chemically synthesized and used to generate a nucleotide sequence of desired length using PCR methods known in the art.

3(a). Expression and Purification Borrelia polypeptides in E. coli

The bacterial expression vector pQE60 is used for bacterial expression of some of the polypeptide fragements of the present invention. (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311). pQE60 encodes ampicillin antibiotic resistance ("Ampr") and contains a bacterial origin of replication ("ori"), an IPTG inducible promoter, a ribosome binding site ("RBS"), six codons encoding histidine residues that allow affinity purification using nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin (QIAGEN, Inc., *supra*) and suitable single restriction enzyme cleavage sites. These elements are arranged such that an inserted DNA fragment encoding a polypeptide expresses that polypeptide with the six His residues (i.e., a "6 X His tag") covalently linked to the carboxyl terminus of that polypeptide.

The DNA sequence encoding the desired portion of a *B. burgdorferi* protein of the present invention is amplified from *B. burgdorferi* genomic DNA using PCR oligonucleotide primers which anneal to the 5' and 3' sequences coding for the portions of the *B. burgdorferi* polynucleotide shown in Table 1. Additional nucleotides containing restriction sites to facilitate cloning in the pQE60 vector are added to the 5' and 3' sequences, respectively.

For cloning the mature protein, the 5' primer has a sequence containing an appropriate restriction site followed by nucleotides of the amino terminal coding sequence of the desired *B. burgdorferi* polynucleotide sequence in Table 1. One of ordinary skill in the art would appreciate that the point in the protein coding sequence where the 5' and 3' primers begin may be varied to amplify a DNA segment encoding any desired portion of the complete protein shorter or longer than the mature form. The 3' primer has a sequence containing an appropriate restriction site

followed by nucleotides complementary to the 3' end of the polypeptide coding sequence of Table 1, excluding a stop codon, with the coding sequence aligned with the restriction site so as to maintain its reading frame with that of the six His codons in the pQE60 vector.

The amplified *B. burgdorferi* DNA fragment and the vector pQE60 are digested with restriction enzymes which recognize the sites in the primers and the digested DNAs are then ligated together. The *B. burgdorferi* DNA is inserted into the restricted pQE60 vector in a manner which places the *B. burgdorferi* protein coding region downstream from the IPTG-inducible promoter and in-frame with an initiating AUG and the six histidine codons.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures such as those described by Sambrook et al., *supra*.. *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses the lac repressor and confers kanamycin resistance ("Kanr"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing a *B. burgdorferi* polypeptide, is available commercially (QIAGEN, Inc., *supra*). Transformants are identified by their ability to grow on LB agar plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/ml) and kanamycin (25 µg/ml). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:25 to 1:250. The cells are grown to an optical density at 600 nm ("OD600") of between 0.4 and 0.6. Isopropyl-β-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from the lac repressor sensitive promoter, by inactivating the lacI repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation.

The cells are then stirred for 3-4 hours at 4°C in 6M guanidine-HCl, pH 8. The cell debris is removed by centrifugation, and the supernatant containing the *B. burgdorferi* polypeptide is loaded onto a nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin column (QIAGEN, Inc., *supra*). Proteins with a 6 x His tag bind to the Ni-NTA resin with high affinity are purified in a simple one-step procedure (for details see: The QIAexpressionist, 1995, QIAGEN, Inc., *supra*). Briefly the supernatant is loaded onto the column in 6 M guanidine-HCl, pH 8, the column is first washed with 10 volumes of 6 M guanidine-HCl, pH 8, then washed with 10 volumes of 6 M guanidine-HCl pH 6, and finally the *B. burgdorferi* polypeptide is eluted with 6 M guanidine-HCl, pH 5.

The purified protein is then renatured by dialyzing it against phosphate-buffered saline (PBS) or 50 mM Na-acetate, pH 6 buffer plus 200 mM NaCl. Alternatively, the protein could be successfully refolded while immobilized on the Ni-NTA column. The recommended conditions are as follows: renature using a linear 6M-1M urea gradient in 500 mM NaCl, 20% glycerol, 20

a period of 1.5 hours or more. After renaturation the proteins can be eluted by the addition of 250 mM immidazole. Immidazole is removed by a final dialyzing step against PBS or 50 mM sodium acetate pH 6 buffer plus 200 mM NaCl. The purified protein is stored at 4° C or frozen at -80° C.

The polypeptide of the present invention are also prepared using a non-denaturing protein purification method. For these polypeptides, the cell pellet from each liter of culture is resuspended in 25 mls of Lysis Buffer A at 4°C (Lysis Buffer A = 50 mM Na-phosphate, 300 mM NaCl, 10 mM 2-mercaptoethanol, 10% Glycerol, pH 7.5 with 1 tablet of Complete EDTA-free protease inhibitor cocktail (Boehringer Mannheim #1873580) per 50 ml of buffer). Absorbance at 550 nm is approximately 10-20 O.D./ml. The suspension is then put through three freeze/thaw cycles from -70°C (using a ethanol-dry ice bath) up to room temperature. The cells are lysed via sonication in short 10 sec bursts over 3 minutes at approximately 80W while kept on ice. The sonicated sample is then centrifuged at 15,000 RPM for 30 minutes at 4°C. The supernatant is passed through a column containing 1.0 ml of CL-4B resin to pre-clear the sample of any proteins that may bind to agarose non-specifically, and the flow-through fraction is collected.

The pre-cleared flow-through is applied to a nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin column (Quiagen, Inc., *supra*). Proteins with a 6 X His tag bind to the Ni-NTA resin with high affinity and can be purified in a simple one-step procedure. Briefly, the supernatant is loaded onto the column in Lysis Buffer A at 4°C, the column is first washed with 10 volumes of Lysis Buffer A until the A280 of the eluate returns to the baseline. Then, the column is washed with 5 volumes of 40 mM Imidazole (92% Lysis Buffer A / 8% Buffer B) (Buffer B = 50 mM Na-Phosphate, 300 mM NaCl, 10% Glycerol, 10 mM 2-mercaptoethanol, 500 mM Imidazole, pH of the final buffer should be 7.5). The protein is eluted off of the column with a series of increasing Imidazole solutions made by adjusting the ratios of Lysis Buffer A to Buffer B. Three different concentrations are used: 3 volumes of 75 mM Imidazole, 3 volumes of 150 mM Imidazole, 5 volumes of 500 mM Imidazole. The fractions containing the purified protein are analyzed using 8 %, 10 % or 14% SDS-PAGE depending on the protein size. The purified protein is then dialyzed 2X against phosphate-buffered saline (PBS) in order to place it into an easily workable buffer. The purified protein is stored at 4° C or frozen at -80°.

The following alternative method may be used to purify *B. burgdorferi* expressed in *E coli* when it is present in the form of inclusion bodies. Unless otherwise specified, all of the following steps are conducted at 4-10°C.

Upon completion of the production phase of the *E. coli* fermentation, the cell culture is cooled to 4-10°C and the cells are harvested by continuous centrifugation at 15,000 rpm (Heraeus Sepatech). On the basis of the expected yield of protein per unit weight of cell paste and the amount of purified protein required, an appropriate amount of cell paste, by weight, is suspended in a buffer solution containing 100 mM Tris, 50 mM EDTA, pH 7.4. The cells are then lysed

The cells are then lysed by passing the solution through a microfluidizer (Microfuidics, Corp. or APV Gaulin, Inc.) twice at 4000-6000 psi. The homogenate is then mixed with NaCl solution to a final concentration of 0.5 M NaCl, followed by centrifugation at 7000 x *g* for 15 min. The resultant pellet is washed again using 0.5M NaCl, 100 mM Tris, 50 mM EDTA, pH 7.4.

The resulting washed inclusion bodies are solubilized with 1.5 M guanidine hydrochloride (GuHCl) for 2-4 hours. After 7000 x *g* centrifugation for 15 min., the pellet is discarded and the *B. burgdorferi* polypeptide-containing supernatant is incubated at 4°C overnight to allow further GuHCl extraction.

Following high speed centrifugation (30,000 x *g*) to remove insoluble particles, the GuHCl solubilized protein is refolded by quickly mixing the GuHCl extract with 20 volumes of buffer containing 50 mM sodium, pH 4.5, 150 mM NaCl, 2 mM EDTA by vigorous stirring. The refolded diluted protein solution is kept at 4°C without mixing for 12 hours prior to further purification steps.

To clarify the refolded *B. burgdorferi* polypeptide solution, a previously prepared tangential filtration unit equipped with 0.16 µm membrane filter with appropriate surface area (e.g., Filtron), equilibrated with 40 mM sodium acetate, pH 6.0 is employed. The filtered sample is loaded onto a cation exchange resin (e.g., Poros HS-50, Perseptive Biosystems). The column is washed with 40 mM sodium acetate, pH 6.0 and eluted with 250 mM, 500 mM, 1000 mM, and 1500 mM NaCl in the same buffer, in a stepwise manner. The absorbance at 280 mm of the effluent is continuously monitored. Fractions are collected and further analyzed by SDS-PAGE.

Fractions containing the *B. burgdorferi* polypeptide are then pooled and mixed with 4 volumes of water. The diluted sample is then loaded onto a previously prepared set of tandem columns of strong anion (Poros HQ-50, Perseptive Biosystems) and weak anion (Poros CM-20, Perseptive Biosystems) exchange resins. The columns are equilibrated with 40 mM sodium acetate, pH 6.0. Both columns are washed with 40 mM sodium acetate, pH 6.0, 200 mM NaCl. The CM-20 column is then eluted using a 10 column volume linear gradient ranging from 0.2 M NaCl, 50 mM sodium acetate, pH 6.0 to 1.0 M NaCl, 50 mM sodium acetate, pH 6.5. Fractions are collected under constant A_{280} monitoring of the effluent. Fractions containing the *B. burgdorferi* polypeptide (determined, for instance, by 16% SDS-PAGE) are then pooled.

The resultant *B. burgdorferi* polypeptide exhibits greater than 95% purity after the above refolding and purification steps. No major contaminant bands are observed from Commassie blue stained 16% SDS-PAGE gel when 5 µg of purified protein is loaded. The purified protein is also tested for endotoxin/LPS contamination, and typically the LPS content is less than 0.1 ng/ml according to LAL assays.

3(b). Alternative Expression and Purification Borrelia polypeptides in E.

coli

Tthe vector pQE10 is alternatively used to clone and express some of the polypeptides of the present invention for use in the soft tissue and systemic infection models discussed below. The difference being such that an inserted DNA fragment encoding a polypeptide expresses that polypeptide with the six His residues (i.e., a "6 X His tag") covalently linked to the amino terminus of that polypeptide. The bacterial expression vector pQE10 (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311) was used in this example . The components of the pQE10 plasmid are arranged such that the inserted DNA sequence encoding a polypeptide of the present invention expresses the polypeptide with the six His residues (*i.e.*, a "6 X His tag")) covalently linked to the amino terminus.

The DNA sequences encoding the desired portions of a polypeptide of Table 1 were amplified using PCR oligonucleotide primers from genomic *B. burgdorferi* DNA. The PCR primers anneal to the nucleotide sequences encoding the desired amino acid sequence of a polypeptide of the present invention. Additional nucleotides containing restriction sites to facilitate cloning in the pQE10 vector were added to the 5' and 3' primer sequences, respectively.

For cloning a polypeptide of the present invention, the 5' and 3' primers were selected to amplify their respective nucleotide coding sequences. One of ordinary skill in the art would appreciate that the point in the protein coding sequence where the 5' and 3' primers begins may be varied to amplify a DNA segment encoding any desired portion of a polypeptide of the present invention. The 5' primer was designed so the coding sequence of the 6 X His tag is aligned with the restriction site so as to maintain its reading frame with that of *B. burgdorferi* polypeptide. The 3' was designed to include an stop codon. The amplified DNA fragment was then cloned, and the protein expressed, as described above for the pQE60 plasmid.

The DNA sequences of Table 1 encoding amino acid sequences may also be cloned and expressed as fusion proteins by a protocol similar to that described directly above, wherein the pET-32b(+) vector (Novagen, 601 Science Drive, Madison, WI 53711) is preferentially used in place of pQE10.

The above methods are not limited to the polypeptide fragements actually produced. The above method, like the methods below, can be used to produce either full length polypeptides or desired fragements therof.

3(c). Alternative Expression and Purification of Borrelia polypeptides in E. coli

The bacterial expression vector pQE60 is used for bacterial expression in this example (QIAGEN, Inc., 9259 Eton Avenue, Chatsworth, CA, 91311). However, in this example, the polypeptide coding sequence is inserted such that translation of the six His codons is prevented and, therefore, the polypeptide is produced with no 6 X His tag.

The DNA sequence encoding the desired portion of the *B. burgdorferi* amino acid sequence is amplified from an *B. burgdorferi* genomic DNA prep the deposited DNA clones

using PCR oligonucleotide primers which anneal to the 5' and 3' nucleotide sequences corresponding to the desired portion of the *B. burgdorferi* polypeptides. Additional nucleotides containing restriction sites to facilitate cloning in the pQE60 vector are added to the 5' and 3' primer sequences.

For cloning a *B. burgdorferi* polypeptides of the present invention, 5' and 3' primers are selected to amplify their respective nucleotide coding sequences. One of ordinary skill in the art would appreciate that the point in the protein coding sequence where the 5' and 3' primers begin may be varied to amplify a DNA segment encoding any desired portion of a polypeptide of the present invention. The 3' and 5' primers contain appropriate restriction sites followed by nucleotides complementary to the 5' and 3' ends of the coding sequence respectively. The 3' primer is additionally designed to include an in-frame stop codon.

The amplified *B. burgdorferi* DNA fragments and the vector pQE60 are digested with restriction enzymes recognizing the sites in the primers and the digested DNAs are then ligated together. Insertion of the *B. burgdorferi* DNA into the restricted pQE60 vector places the *B. burgdorferi* protein coding region including its associated stop codon downstream from the IPTG-inducible promoter and in-frame with an initiating AUG. The associated stop codon prevents translation of the six histidine codons downstream of the insertion point.

The ligation mixture is transformed into competent *E. coli* cells using standard procedures such as those described by Sambrook et al. *E. coli* strain M15/rep4, containing multiple copies of the plasmid pREP4, which expresses the lac repressor and confers kanamycin resistance ("Kanr"), is used in carrying out the illustrative example described herein. This strain, which is only one of many that are suitable for expressing *B. burgdorferi* polypeptide, is available commercially (QIAGEN, Inc., *supra*). Transformants are identified by their ability to grow on LB plates in the presence of ampicillin and kanamycin. Plasmid DNA is isolated from resistant colonies and the identity of the cloned DNA confirmed by restriction analysis, PCR and DNA sequencing.

Clones containing the desired constructs are grown overnight ("O/N") in liquid culture in LB media supplemented with both ampicillin (100 µg/ml) and kanamycin (25 µg/ml). The O/N culture is used to inoculate a large culture, at a dilution of approximately 1:25 to 1:250. The cells are grown to an optical density at 600 nm ("OD600") of between 0.4 and 0.6. isopropyl-b-D-thiogalactopyranoside ("IPTG") is then added to a final concentration of 1 mM to induce transcription from the *lac* repressor sensitive promoter, by inactivating the lacI repressor. Cells subsequently are incubated further for 3 to 4 hours. Cells then are harvested by centrifugation.

To purify the *B. burgdorferi* polypeptide, the cells are then stirred for 3-4 hours at 4°C in 6M guanidine-HCl, pH 8. The cell debris is removed by centrifugation, and the supernatant containing the *B. burgdorferi* polypeptide is dialyzed against 50 mM Na-acetate buffer pH 6, supplemented with 200 mM NaCl. Alternatively, the protein can be successfully refolded by

inhibitors. After renaturation the protein can be purified by ion exchange, hydrophobic interaction and size exclusion chromatography. Alternatively, an affinity chromatography step such as an antibody column can be used to obtain pure *B. burgdorferi* polypeptide. The purified protein is stored at 4° C or frozen at -80° C.

The following alternative method may be used to purify *B. burgdorferi* polypeptides expressed in *E coli* when it is present in the form of inclusion bodies. Unless otherwise specified, all of the following steps are conducted at 4-10°C.

Upon completion of the production phase of the *E. coli* fermentation, the cell culture is cooled to 4-10°C and the cells are harvested by continuous centrifugation at 15,000 rpm (Heraeus Sepatech). On the basis of the expected yield of protein per unit weight of cell paste and the amount of purified protein required, an appropriate amount of cell paste, by weight, is suspended in a buffer solution containing 100 mM Tris, 50 mM EDTA, pH 7.4. The cells are dispersed to a homogeneous suspension using a high shear mixer.

The cells ware then lysed by passing the solution through a microfluidizer (Microfuidics, Corp. or APV Gaulin, Inc.) twice at 4000-6000 psi. The homogenate is then mixed with NaCl solution to a final concentration of 0.5 M NaCl, followed by centrifugation at 7000 x *g* for 15 min. The resultant pellet is washed again using 0.5M NaCl, 100 mM Tris, 50 mM EDTA, pH 7.4.

The resulting washed inclusion bodies are solubilized with 1.5 M guanidine hydrochloride (GuHCl) for 2-4 hours. After 7000 x *g* centrifugation for 15 min., the pellet is discarded and the *B. burgdorferi* polypeptide-containing supernatant is incubated at 4°C overnight to allow further GuHCl extraction.

Following high speed centrifugation (30,000 x *g*) to remove insoluble particles, the GuHCl solubilized protein is refolded by quickly mixing the GuHCl extract with 20 volumes of buffer containing 50 mM sodium, pH 4.5, 150 mM NaCl, 2 mM EDTA by vigorous stirring. The refolded diluted protein solution is kept at 4°C without mixing for 12 hours prior to further purification steps.

To clarify the refolded *B. burgdorferi* polypeptide solution, a previously prepared tangential filtration unit equipped with 0.16 µm membrane filter with appropriate surface area (e.g., Filtron), equilibrated with 40 mM sodium acetate, pH 6.0 is employed. The filtered sample is loaded onto a cation exchange resin (e.g., Poros HS-50, Perseptive Biosystems). The column is washed with 40 mM sodium acetate, pH 6.0 and eluted with 250 mM, 500 mM, 1000 mM, and 1500 mM NaCl in the same buffer, in a stepwise manner. The absorbance at 280 mm of the effluent is continuously monitored. Fractions are collected and further analyzed by SDS-PAGE.

Fractions containing the *B. burgdorferi* polypeptide are then pooled and mixed with 4 volumes of water. The diluted sample is then loaded onto a previously prepared set of tandem columns of strong anion (Poros HQ-50, Perseptive Biosystems) and weak anion (Poros CM-20,

Perseptive Biosystems) exchange resins. The columns are equilibrated with 40 mM sodium acetate, pH 6.0. Both columns are washed with 40 mM sodium acetate, pH 6.0, 200 mM NaCl. The CM-20 column is then eluted using a 10 column volume linear gradient ranging from 0.2 M NaCl, 50 mM sodium acetate, pH 6.0 to 1.0 M NaCl, 50 mM sodium acetate, pH 6.5. Fractions are collected under constant A_{280} monitoring of the effluent. Fractions containing the *B. burgdorferi* polypeptide (determined, for instance, by 16% SDS-PAGE) are then pooled.

The resultant *B. burgdorferi* polypeptide exhibits greater than 95% purity after the above refolding and purification steps. No major contaminant bands are observed from Commassie blue stained 16% SDS-PAGE gel when 5 μg of purified protein is loaded. The purified protein is also tested for endotoxin/LPS contamination, and typically the LPS content is less than 0.1 ng/ml according to LAL assays.

3(d). Cloning and Expression of B. burgdorferi in Other Bacteria

B. burgdorferi polypeptides can also be produced in: *B. burgdorferi* using the methods of S. Skinner et al., (1988) Mol. Microbiol. 2:289-297 or J. I. Moreno (1996) Protein Expr. Purif. 8(3):332-340; *Lactobacillus* using the methods of C. Rush et al., 1997 Appl. Microbiol. Biotechnol. 47(5):537-542; or in *Bacillus subtilis* using the methods Chang et al., U.S. Patent No. 4,952,508.

4. Cloning and Expression in COS Cells

A *B. burgdorferi* expression plasmid is made by cloning a portion of the DNA encoding a *B. burgdorferi* polypeptide into the expression vector pDNAI/Amp or pDNAIII (which can be obtained from Invitrogen, Inc.). The expression vector pDNAI/amp contains: (1) an *E. coli* origin of replication effective for propagation in *E. coli* and other prokaryotic cells; (2) an ampicillin resistance gene for selection of plasmid-containing prokaryotic cells; (3) an SV40 origin of replication for propagation in eukaryotic cells; (4) a CMV promoter, a polylinker, an SV40 intron; (5) several codons encoding a hemagglutinin fragment (i.e., an "HA" tag to facilitate purification) followed by a termination codon and polyadenylation signal arranged so that a DNA can be conveniently placed under expression control of the CMV promoter and operably linked to the SV40 intron and the polyadenylation signal by means of restriction sites in the polylinker. The HA tag corresponds to an epitope derived from the influenza hemagglutinin protein described by Wilson et al. 1984 Cell 37:767. The fusion of the HA tag to the target protein allows easy detection and recovery of the recombinant protein with an antibody that recognizes the HA epitope. pDNAIII contains, in addition, the selectable neomycin marker.

A DNA fragment encoding a *B. burgdorferi* polypeptide is cloned into the polylinker region of the vector so that recombinant protein expression is directed by the CMV promoter. The plasmid construction strategy is as follows. The DNA from a *B. burgdorferi* genomic DNA prep is amplified using primers that contain convenient restriction sites, much as described above for

construction of vectors for expression of *B. burgdorferi* in *E. coli*. The 5' primer contains a Kozak sequence, an AUG start codon, and nucleotides of the 5' coding region of the *B. burgdorferi* polypeptide. The 3' primer, contains nucleotides complementary to the 3' coding sequence of the *B. burgdorferi* DNA, a stop codon, and a convenient restriction site.

The PCR amplified DNA fragment and the vector, pDNAI/Amp, are digested with appropriate restriction enzymes and then ligated. The ligation mixture is transformed into an appropriate *E. coli* strain such as SURE™ (Stratagene Cloning Systems, La Jolla, CA 92037), and the transformed culture is plated on ampicillin media plates which then are incubated to allow growth of ampicillin resistant colonies. Plasmid DNA is isolated from resistant colonies and examined by restriction analysis or other means for the presence of the fragment encoding the *B. burgdorferi* polypeptide

For expression of a recombinant *B. burgdorferi* polypeptide, COS cells are transfected with an expression vector, as described above, using DEAE-dextran, as described, for instance, by Sambrook et al. (*supra*). Cells are incubated under conditions for expression of *B. burgdorferi* by the vector.

Expression of the *B. burgdorferi*-HA fusion protein is detected by radiolabeling and immunoprecipitation, using methods described in, for example Harlow et al., *supra*.. To this end, two days after transfection, the cells are labeled by incubation in media containing ^{35}S-cysteine for 8 hours. The cells and the media are collected, and the cells are washed and the lysed with detergent-containing RIPA buffer: 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% NP-40, 0.5% DOC, 50 mM TRIS, pH 7.5, as described by Wilson et al. (*supra*). Proteins are precipitated from the cell lysate and from the culture media using an HA-specific monoclonal antibody. The precipitated proteins then are analyzed by SDS-PAGE and autoradiography. An expression product of the expected size is seen in the cell lysate, which is not seen in negative controls.

5. Cloning and Expression in CHO Cells

The vector pC4 is used for the expression of *B. burgdorferi* polypeptide in this example. Plasmid pC4 is a derivative of the plasmid pSV2-dhfr (ATCC Accession No. 37146). The plasmid contains the mouse DHFR gene under control of the SV40 early promoter. Chinese hamster ovary cells or other cells lacking dihydrofolate activity that are transfected with these plasmids can be selected by growing the cells in a selective medium (alpha minus MEM, Life Technologies) supplemented with the chemotherapeutic agent methotrexate. The amplification of the DHFR genes in cells resistant to methotrexate (MTX) has been well documented. *See, e.g.*, Alt et al., 1978, J. Biol. Chem. 253:1357-1370; Hamlin et al., 1990, Biochem. et Biophys. Acta, 1097:107-143; Page et al., 1991, Biotechnology 9:64-68. Cells grown in increasing concentrations of MTX develop resistance to the drug by overproducing the target enzyme, DHFR, as a result of amplification of the DHFR gene. If a second gene is linked to the DHFR gene, it is usually co-amplified and over-expressed. It is known in the art that this approach may

be used to develop cell lines carrying more than 1,000 copies of the amplified gene(s). Subsequently, when the methotrexate is withdrawn, cell lines are obtained which contain the amplified gene integrated into one or more chromosome(s) of the host cell.

Plasmid pC4 contains the strong promoter of the long terminal repeat (LTR) of the Rouse Sarcoma Virus, for expressing a polypeptide of interest, Cullen, et al. (1985) Mol. Cell. Biol. 5:438-447; plus a fragment isolated from the enhancer of the immediate early gene of human cytomegalovirus (CMV), Boshart, et al., 1985, Cell 41:521-530. Downstream of the promoter are the following single restriction enzyme cleavage sites that allow the integration of the genes: *Bam* HI, *Xba* I, and *Asp* 718. Behind these cloning sites the plasmid contains the 3' intron and polyadenylation site of the rat preproinsulin gene. Other high efficiency promoters can also be used for the expression, e.g., the human ß-actin promoter, the SV40 early or late promoters or the long terminal repeats from other retroviruses, e.g., HIV and HTLVI. Clontech's Tet-Off and Tet-On gene expression systems and similar systems can be used to express the *B. burgdorferi* polypeptide in a regulated way in mammalian cells (Gossen et al., 1992, Proc. Natl. Acad. Sci. USA 89:5547-5551. For the polyadenylation of the mRNA other signals, e.g., from the human growth hormone or globin genes can be used as well. Stable cell lines carrying a gene of interest integrated into the chromosomes can also be selected upon co-transfection with a selectable marker such as gpt, G418 or hygromycin. It is advantageous to use more than one selectable marker in the beginning, e.g., G418 plus methotrexate.

The plasmid pC4 is digested with the restriction enzymes and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel. The DNA sequence encoding the *B. burgdorferi* polypeptide is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' sequences of the desired portion of the gene. A 5' primer containing a restriction site, a Kozak sequence, an AUG start codon, and nucleotides of the 5' coding region of the *B. burgdorferi* polypeptide is synthesized and used. A 3' primer, containing a restriction site, stop codon, and nucleotides complementary to the 3' coding sequence of the *B. burgdorferi* polypeptides is synthesized and used. The amplified fragment is digested with the restriction endonucleases and then purified again on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC4 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene are used for transfection. Five µg of the expression plasmid pC4 is cotransfected with 0.5 µg of the plasmid pSVneo using a lipid-mediated transfection agent such as Lipofectin™ or LipofectAMINE.™ (LifeTechnologies Gaithersburg, MD). The plasmid pSV2-neo contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418

MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 μM, 2 μM, 5 μM, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100-200 μM. Expression of the desired gene product is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

6. Immunization and Detection of Immune Responses

6(a). B. burgdorferi propagation

B. burgdorferi sensu stricto isolate B31 is propagated in tightly-closed containers at 34°C in modified Barbour-Stoenner-Kelly (BSKII) medium (Barbour, A.G., *Yale J. Biol. Med. 57*:521-525 (1984)) overlaid with a 5%O_2/5%CO_2/90%N_2 gas mixture. Cell densities of these cultures are determined by darkfield microscopy at 400X.

Immunization of Mice and Challenge with B. burgdorferi. For active immunizations BALB/cByJ mice (BALB, Jackson Laboratories) are injected intraperitoneally (i.p.) at week 0 with 20 g of recombinant borrelial protein, or phosphate-buffered saline (PBS), emulsified with complete Freund's adjuvant (CFA), given a similar booster immunization in incomplete Freund's adjuvant (IFA) at week 4, and challenged at week 6. For challenge *B. burgdorferi* are diluted in BSKII from exponentially-growing cultures and mice are injected subcutaneously (s.c.) at the base of the tail with 0.1 ml of these dilutions (typically 10^3-10^4 borreliae; approximately 10-100 times the median infectious dose). Borreliae used for challenge are passaged fewer than six times *in vitro*. To assess infection, mice are sacrificed at 14-17 days post-challenge, and specimens derived from ear, bladder, and tibiotarsal joints are placed in BSKII plus 1.4% gelatin, 13 g/ml amphotericin B, 1.5 g/ml phosphomycin, and 15 g/ml rifampicin, and borrelia outgrowth at two or three weeks is quantified by darkfield microscopy. Batches of BSKII are qualified for infection testing by confirming that they supported the growth of 1-5 cells of isolate B31. In some instances seroconversion for protein P39 reactivity is also used to confirm infections (see below). Others have previously shown that mice elicited antibodies to P39 when inoculated with live borreliae by syringe or tick bite, but not with killed borreliae (Simpson, W.J., *et al., J. Clin. Microbiol. 29*:236-243 (1991)).

6(b). Immunoassays

Several immunoassay formats are used to quantify levels of borrelia-specific antibodies (ELISA and immunoblot), and to evaluate the functional properties of these antibodies (growth inhibition assay). The ELISA and immunoblot assays are also used to detect and quantify antibodies elicited in response to borrelial infection that react with specific borrelial antigens. Where antibodies to certain borrelial antigens are elicited by infection this is taken as evidence that

the borrelial proteins in question are expressed *in vivo*. Absence of infection-derived antibodies (seroconversion) following borrelial challenge is evidence that infection is prevented or suppressed. The immunoblot assay is also used to ascertain whether antibodies raised against recombinant borrelial antigens recognize a protein of similar size in extracts of whole borreliae. Where the natural protein is of similar, or identical, size in the immunoblot assay to the recombinant version of the same protein, this is taken as evidence that the recombinant protein is the product of a full-length clone of the respective gene.

Enzyme-Linked Immunosorbant Assay (ELISA). The ELISA is used to quantify levels of antibodies reactive with borrelial antigens elicited in response to immunization with these borrelial antigens. Wells of 96 well microtiter plates (Immunlon 4, Dynatech, Chantilly, Virginia, or equivalent) are coated with antigen by incubating 50 l of 1 g/ml protein antigen solution in a suitable buffer, typically 0.1 M sodium carbonate buffer at pH 9.6. After decanting unbound antigen, additional binding sites are blocked by incubating 100 l of 3% nonfat milk in wash buffer (PBS, 0.2% Tween 20, pH 7.4). After washing, duplicate serial two-fold dilutions of sera in PBS, Tween 20, 1% fetal bovine serum, are incubated for 1 hr, removed, wells are washed three times, and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG. After three washes, bound antibodies are detected with H_2O_2 and 2,2'-azino-di-(3-ethylbenzthiazoline sulfonate) (Schwan, T.G., *et al., Proc. Natl. Acad. Sci. USA 92*:2909-2913 (1985)) (ABTS®, Kirkegaard & Perry Labs., Gaithersburg, MD) and A_{405} is quantified with a Molecular Devices, Corp. (Menlo Park, California) Vmax™ plate reader. IgG levels twice the background level in serum from naive mice are assigned the minimum titer of 1:100.

6(c). In Vitro Growth Inhibition Assay

Unlike other bacteria, borreliae can be killed by the binding of specific antibodies to their surface antigens. The mechanism for this *in vitro* killing or growth-inhibitory effect is not known, but can occur in the absence of serum complement, or other immune effector functions. Antibodies elicited in animals receiving immunizations with specific borrelial antigens that result in protection from borrelial challenge usually will directly kill borreliae *in vitro*. Thus, the *in vitro* growth inhibition assay also has a high predictive value for the protective potency of the borrelial antibodies, although exceptions, such as antibodies against OspC which are weak at *in vitro* growth inhibition, have been observed. Also, this assay can be used to evaluate the serologic conservation of epitope binding protective antibodies. A microwell antibody titration assay (Sadziene, A., *et al., J. Infect. Dis. 167*:165-172 (1993)) is used to evaluate the growth inhibition (GI) properties of antisera against recombinant borrelial antigens against the homologous B31 isolate, and against various strains of borrelia. Briefly, 10^5 borrelia in 100 l BSKII are added to serial two-fold dilutions of sera in 100 l BSKII in 96-well plates, and the plates are covered and incubated at 34°C in a $5\%O_2/5\%CO_2/90\%N_2$ gas mixture for 72 h prior to quantification of borrelia growth by darkfield microscopy.

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6(d). Sodiumdodecylsulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblotting

Using a single well format, total borrelial protein extracts, recombinant borrelial antigen, or recombinant P39 samples (2 g of purified protein, or more for total borrelial extracts) are boiled in SDS/2-ME sample buffer before electrophoresis through 3% acrylamide stacking gels, and resolving gels of higher acrylamide concentration, typically 10-15% acrylamide monomer. Gels are electro-blotted to nitrocellulose membranes and lanes are probed with dilutions of antibody to be tested for reactivity with specific borrelial antigens, followed by the appropriate secondary antibody-enzyme (horseradish peroxidase) conjugate. When it is desirable to confirm that the protein had transferred following electro-blotting, membranes are stained with Ponceau S. Immunoblot signals from bound antibodies are detected on x-ray film as chemiluminescence using ECL™ reagents (Amersham Corp., Arlington Heights, Illinois).

6(e). Detection of Borrelia mRNA expression

Northern blot analysis is carried out using methods described by, among others, Sambrook *et al., supra.* to detect the expression of the *B. burgdorferi* nucleotide sequences of the present invention in animal tissues. A cDNA probe containing an entire nucleotide sequence shown in Table 1 is labeled with ^{32}P using the *redi*prime™ DNA labeling system (Amersham Life Science), according to manufacturer's instructions. After labeling, the probe is purified using a CHROMA SPIN-100™ column (Clontech Laboratories, Inc.), according to manufacturer's protocol number PT1200-1. The purified labeled probe is then used to detect the expression of *Borrelia* mRNA in an animal tissue sample.

Animal tissues, such as blood or spinal fluid, are examined with the labeled probe using ExpressHyb™ hybridization solution (Clontech) according to manufacturer's protocol number PT1190-1. Following hybridization and washing, the blots are mounted and exposed to film at -70 C overnight, and films developed according to standard procedures.

The disclosure of all publications (including patents, patent applications, journal articles, laboratory manuals, books, or other documents) cited herein are hereby incorporated by reference in their entireties.

The present invention is not to be limited in scope by the specific embodiments described herein, which are intended as single illustrations of individual aspects of the invention. Functionally equivalent methods and components are within the scope of the invention, in addition to those shown and described herein and will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

Provisional Application Serial No. 60/057,483 filed 3 September 1997 is incorporated by reference herein in its entirety.

DEMANDES OU BREVETS VOLUMINEUX

LA PRÉSENTE PARTIE DE CETTE DEMANDE OU CE BREVET COMPREND PLUS D'UN TOME.

CECI EST LE TOME 1 DE 2

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JUMBO APPLICATIONS/PATENTS

THIS SECTION OF THE APPLICATION/PATENT CONTAINS MORE THAN ONE VOLUME

THIS IS VOLUME ____ OF ____

NOTE: For additional volumes-please contact the Canadian Patent Office

What Is Claimed Is:

1. An isolated nucleic acid molecule comprising a polynucleotide having a nucleotide sequence selected from the group consisting of:

(a) a nucleotide sequence encoding any one of the amino acid sequences of the polypeptides shown in Table 1; or

(b) a nucleotide sequence complementary to any one of the nucleotide sequences in (a).

(c) a nucleotide sequence at least 95% identical to any one of the nucleotide sequences shown in Table 1; or,

(d) a nucleotide sequence at least 95% identical to a nucleotide sequence complementary to any one of the nucleotide sequences shown in Table 1.

2. An isolated nucleic acid molecule of claim 1 comprising a polynucleotide which hybridizes under stringent hybridization conditions to a polynucleotide having a nucleotide sequence identical to a nucleotide sequence in (a) or (b) of claim 1.

3. An isolated nucleic acid molecule of claim 1 comprising a polynucleotide which encodes an epitope-bearing portion of a polypeptide in (a) of claim 1.

4. The isolated nucleic acid molecule of claim 3, wherein said epitope-bearing portion of a polypeptide comprises an amino acid sequence listed in Table 4.

5. A method for making a recombinant vector comprising inserting an isolated nucleic acid molecule of claim 1 into a vector.

6. A recombinant vector produced by the method of claim 5.

7. A host cell comprising the vector of claim 6.

8. A method of producing a polypeptide comprising:

(a) growing the host cell of claim 7 such that the protein is expressed by the cell; and

(b) recovering the expressed polypeptide.

9. An isolated polypeptide comprising a polypeptide selected from the group consisting of:

(a) a polypeptide consisting of one of the complete amino acid sequences of Table 1;
(b) a polypeptide consisting of one the complete amino acid sequences of Table 1 except the N-terminal residue;
(c) a fragment of the polypeptide of (a) having biological activity; and
(d) a fragment of the polypeptide of (a) which binds to an antibody specific for the polypeptide of (a).

10. An isolated antibody specific for the polypeptide of claim 9.

11. A polypeptide produced according to the method of claim 8.

12. An isolated polypeptide comprising an amino acid sequence at least 95% identical to a sequence selected from the group consisting of an amino acid sequence of any one of the polypeptides in Table 1.

13. An isolated polypeptide antigen comprising an amino acid sequence of an *B. burgdorferi* epitope shown in Table 4.

14. An isolated nucleic acid molecule comprising a polynucleotide with a nucleotide sequence encoding a polypeptide of claim 9.

15. A hybridoma which produces an antibody of claim 10.

16. A vaccine, comprising:
(1) one or more *B. burgdorferi* polypeptides selected from the group consisting of a polypeptide of claim 9; and
(2) a pharmaceutically acceptable diluent, carrier, or excipient;
wherein said polypeptide is present, in an amount effective to elicit protective antibodies in an animal to a member of the *Borrelia* genus.

17. A method of preventing or attenuating an infection caused by a member of the *Borrelia* genus in an animal, comprising administering to said animal a polypeptide of claim 9, wherein said polypeptide is administered in an amount effective to prevent or attenuate said infection.

18. A method of detecting *Borrelia* nucleic acids in a biological sample comprising:
(a) contacting the sample with one or more nucleic acids of claim 1, under conditions such that hybridization occurs, and
(b) detecting hybridization of said nucleic acids to the one or more *Borrelia* nucleic acid

sequences present in the biological sample.

19. A method of detecting *Borrelia* nucleic acids in a biological sample obtained from an animal, comprising:

(a) amplifying one or more *Borrelia* nucleic acid sequences in said sample using polymerase chain reaction, and

(b) detecting said amplified *Borrelia* nucleic acid.

20. A kit for detecting *Borrelia* antibodies in a biological sample obtained from an animal, comprising

(a) a polypeptide of claim 9 attached to a solid support; and

(b) detecting means.

21. A method of detecting *Borrelia* antibodies in a biological sample obtained from an animal, comprising

(a) contacting the sample with a polypeptide of claim 9; and

(b) detecting antibody-antigen complexes.