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26. Telomeric tracts (60 ng) that contained a Bcl I cohesive end were prepared by annealing two complementary gel-purified oligonucleotides and were subsequently ligated to a Bam HI–linearized replicating plasmid, YRp17 (1 μg). The DNA was then ligated to a Bam HI fragment that contained the *HIS3* gene, the ends of which had been previously filled in with the Klenow fragment of DNA polymerase I, and transformed into the *Escherichia coli* strain HB101. The vectors obtained contained YRp17 flanked by artificial telomeres that were separated by the *HIS3* gene to avoid the rearrangement of inverted repeats in *E. coli* [A. Murray and J. Szostak, *Mol Cell Biol* **6**, 3166 (1986)]. Because ligation of the *HIS3*-containing fragment to the artificial telomeres recreated the Bam HI site, digestion of this plasmid with Bam HI resulted in the formation of a YRp17 plasmid terminated by telomeric sequences. The sequences present at the plasmid termini were identical to those present in the original oligonucleotide with the exception of a 4-bp 5′ overhang.
27. In the case of AT12, one of the two telomeric tracts (*URA3* proximal) contained an additional C to T change, designated by C/T, outside of the two nonoverlapping RAP1 binding sites. In the case of AT16, an apparent structural anomaly at the end of the *URA3* proximal telomere did not permit an accurate determination of the terminal 5 bp of this telomere.
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33. We thank P. Carrico, S. Marglin, and L. Chen-Chiu for technical assistance; B. Rapkin for guidance with statistical analysis; S. Jinks-Robertson for providing plasmids; D. Gottschling for communicating results before publication; and H. Klein, A. Mitchell, M. A. Olsey, L. Sussel, M. Gwadz, B. Dunn, and E. B. Hoffman for critical comments. Supported by American Cancer Society grant MV-446 (to A.J.L.); by NIH grant GM 40094 and the Searle Scholars' Program Chicago Community Trust (to D.S.); and by a postdoctoral fellowship from the Jane Coffin Childs Memorial Fund (to S.K.)

7 May 1990; accepted 10 August 1990

Protection of Mice Against the Lyme Disease Agent by Immunizing with Recombinant OspA

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Lyme borreliosis is a tick-borne illness caused by *Borrelia burgdorferi*. The gene for outer surface protein A (OspA) from *B. burgdorferi* strain N40 was cloned into an expression vector and expressed in *Escherichia coli*. C3H/HeJ mice actively immunized with live transformed *E. coli* or purified recombinant OspA protein produced antibodies to OspA and were protected from challenge with several strains of *B. burgdorferi*. Recombinant OspA is a candidate for a vaccine for Lyme borreliosis.

LYME DISEASE IS A MULTISYSTEM SPIrochetal illness characterized by rheumatologic, neurologic, cardiac, and dermatologic manifestations. The causative agent, *Borrelia burgdorferi*, is transmitted to humans mostly by *Ixodes* ticks (*1*). The host immune response to *B. burgdorferi* is incompletely understood; however, humoral immunity is important in protecting animals from infection. Passive immunization of hamsters with antiserum to *B. burgdorferi* prevents infection from intraperitoneal challenge with *B. burgdorferi* (*2*). The protection is partially strain-specific, however, because antiserum to *B. burgdorferi* strains from different geographic locations does not show full cross protection (*3*). Active immunization of hamsters with whole inactivated *B. burgdorferi* strain 297 prevents subsequent infection with *B. burgdorferi* strain 297 (*4*). The hamster model, however, is limited for the study of Lyme borreliosis because the animals do not develop clinical manifestations of Lyme disease.

A model of Lyme borreliosis has been developed in immunocompetent mice that resembles some of the clinical manifestations of Lyme disease in humans (*5*). A strain of *B. burgdorferi*, designated N40, was isolated from Westchester County, New York; C3H/He mice infected with *B. burgdorferi* N40 develop spirochetemia 5 days after challenge and peak arthritis and carditis 14 days after infection. This model is useful for active immunization experiments with *B. burgdorferi* in contrast to the model of Lyme borreliosis in severe combined immunodeficiency mice (*6*).

We first verified that passive immunization is protective in the C3H/He mouse model. Mice were passively immunized with antiserum to *B. burgdorferi* N40 from mouse or rabbit and then challenged with *B. burgdorferi* N40 (*7*). Control groups were immunized with normal mouse or rabbit serum. After 5 or 14 days cultures of the blood and spleen were incubated for 2 weeks in Barbour-Stoenner-Kelly (BSK) medium (*8*). As expected from results in the hamster model, passive immunity was protective. Furthermore, the protective effect of rabbit serum was maintained at a dilution of 1:500. Protection extended to *B. burgdorferi* strain B31 and the clinical manifestations of disease at 14 days was prevented (Table 1).

The antigens responsible for eliciting the production of protective antibodies are not known; however, several outer surface proteins on *B. burgdorferi* are candidates. The outer membrane of *B. burgdorferi* is coated with a 31-kD protein known as outer surface protein A (OspA) (*9*), and a 34-kD protein named outer protein B (OspB) (*10*).

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These proteins are heterogenous, particularly OspB, among *B. burgdorferi* isolates, but the degree of variability has not been clarified (*11*). In addition, a 41-kD protein is associated with the *B. burgdorferi* flagellum (*12*). The genes for these proteins have been cloned and sequenced from *B. burgdorferi* B31 (*13, 15*). During human infection, antibodies are produced to the 41-kD flagellar antigen early in disease and to OspA and OspB, among other proteins, later in the illness (*15*). In the mouse model antibodies to these three antigens occur within the first month of illness (*5*). We now show that active immunization with recombinant OspA from *B. burgdorferi* N40 protects mice from subsequent infection with *B. burgdorferi* strains N40, B31, and CD16.

Table 1. Protection of CeH/HeJ mice from infection with *B. burgdorferi* N40 or B31 by passive immunization using polyclonal antiserum to *B. burgdorferi* N40. Groups of five mice were passively immunized with antiserum (0.1 ml). Control groups were immunized with normal serum. One day after immunization, mice were intradermally injected with 10^4 *B. burgdorferi* N40 or B31 spirochetes. At 5 or 14 days organs were harvested, and one or two cultures of each organ were incubated for 2 weeks and examined by dark-field microscopy. Twenty high-power fields were scanned per culture. Positive cultures had between 1 and 100 spirochetes while negative cultures had no organisms. The table reports results of blood and splenic cultures (number of positive cultures/total number of cultures) begun 5 or 14 days after challenge and incubated for 2 weeks and the histopathologic evaluation of the heart and joints 14 days after infection.

Antiserum (dilution)	Cultures				Histopathology	
	Blood		Spleen		Arthritis	Carditis
	5 day	14 day	5 day	14 day	14 day	14 day
Mice challenged with N40						
Mouse anti–*B. burgdorferi* N40 (1:5)	0/8		0/8			
Normal mouse serum (1:5)	8/8		8/8			
Rabbit anti–*B. burgdorferi* N40 (1:5)	0/10	0/10	0/5	0/10	0/10	0/10
Rabbit anti–*B. burgdorferi* N40 (1:50)	0/10		0/5			
Rabbit anti–*B. burgdorferi* N40 (1:500)	0/10		0/5			
Normal rabbit serum (1:5)	9/10	8/10	8/10	6/9	10/10	10/10
Mice challenged with B31						
Rabbit anti–*B. burgdorferi* N40 (1:5)		0/5		0/5	0/5	0/5
Normal rabbit serum (1:5)		5/5		4/5	5/5	5/5

Table 2. Protection of mice from infection with *B. burgdorferi* by active immunization with *E. coli* (5×10^6) expressing OspA from *B. burgdorferi* N40 or purified recombinant OspA fusion protein, and passive immunization with serum from mice actively immunized with the OspA expressing *E. coli* or monoclonal antibodies to OspA. Groups of five C3H/HeJ mice were actively immunized intraperitoneally with live *E. coli* expressing recombinant OspA from *B. burgdorferi* N40, or with *E. coli* lacking the OspA product. Mice were actively immunized subcutaneously with purified recombinant OspA-glutathione *S*-transferase fusion protein (20 μg) or purified glutathione *S*-transferase in complete Freund's adjuvant and boosted weekly for 2 weeks with the equivalent amount of protein in incomplete Freund's adjuvant. All mice were then injected with *B. burgdorferi* strain N40, B31, or CD16 spirochetes (10^4) intradermally and examined at 5 or 14 days. The antiserum for passive immunization was drawn from mice actively immunized with *E. coli* expressing recombinant OspA. Serum (0.1 ml), either undiluted or diluted 1:5, was used to passively immunize mice. The supernatant (0.1 ml) from cells expressing monoclonal antibodies (MAbs) to OspA was administered undiluted. The mice were injected the next day with *B. burgdorferi* N40 and examined at 5 days. Cultures were incubated and examined as described in Table 1.

Immunization	*Borrelia* strain	Cultures				Histopathology	
		Blood		Spleen		Arthritis	Carditis
		5 day	14 day	5 day	14 days	14 day	15 day
		Active					
E. coli expressing OspA	N40	0/10	0/5	0/10		2/5	2/5
	B31	0/5		0/5			
	CD16	0/5					
E. coli lacking OspA	N40	9/9	5/5	7/9		5/5	5/5
	B31	5/5		5/5			
	CD16	3/5					
OspA fusion protein	N40		0/5		0/5	0/9	0/10
Glutathione *S*-transferase	N40		5/5		3/5	10/10	10/10
		Passive					
E. coli expressing OspA (undil.)	N40	0/4					
E. coli expressing OspA (1:5)	N40	0/4					
IgG3 OspA MAb	N40	0/5					
IgG1 OspA MAb	N40	5/5					
Normal rabbit serum (1:5)	N40	8/10					

The gene for OspA from *B. burgdorferi* N40 was amplified with genomic DNA from *B. burgdorferi* N40 (*16*) and the polymerase chain reaction (PCR), and cloned in *E. coli* (*17*). The gene for OspA-N40 was sequenced and found to be 819 nucleotides (*18*). The sequence was compared to the published sequences for *B. burgdorferi* OspA, including strain B31 (*13*) and strain ZS7, an isolate from Freiburg, Germany (*19*). The OspA-N40 gene differs from OspA-B31 at two nucleotide positions, 117 and 446. This changes Lys^{39} to Asn^{39} and Gly^{149} to Glu^{149} OspA-N40 differs from OspA-ZS7 by one nucleotide at position 490, which changes Ser^{164} to Gly^{164}. These comparisons suggest that OspA is conserved among different isolates.

Escherichia coli transformed with 197-OspA-N40, a recombinant plasmid that directs the synthesis of OspA-N40 by the phage lambda PL promoter, were induced to express recombinant OspA-N40 by growth at 42°C for 2 hours. Upon induction a new band appeared on SDS–polyacrylamide gel electrophoresis accounting for approximately 5% of the total cell protein. Sequencing of the NH_2-terminus of the new protein revealed that the first 15 amino acids corresponded to the amino acid sequence predicted from the OspA-N40 gene sequence, identifying the protein as OspA-N40. The NH_2-terminal amino acid sequence was Met-X-Lys-Tyr-Leu-Leu-Gly-Ile-Gly-Leu-Ile-Leu-Ala-Leu-Ile. An immunoblot of a cell and an osmotic shock (*20*) extract of *E. coli* expressing OspA-N40 stained with anti *B. burgdorferi* N40 mouse serum or with a monoclonal antibody to OspA-N40 showed that OspA-N40 was expressed and localized at least in part to the periplasmic space (*21*).

C3H/He mice were then immunized to determine if an immune response to recombinant OspA could be elicited. Groups of five mice were injected intraperitoneally once weekly with 5×10^6 live *E. coli* expressing OspA-N40 for 3 weeks. Control mice were immunized with *E. coli* transformed with pDC197-12. An antibody response to OspA-N40 was elicited by the fourth week, and could be detected by immunoblot (Fig. 1), to a dilution of 1:1000.

During the fifth week the mice were challenged with *B. burgdorferi* to determine if active immunization would elicit a protective immune response. Mice actively immunized with *E. coli* expressing OspA-N40 were infected intradermally with 10^4 *B. burgdorferi* N40 and examined at 5 or 14 days. The control group consisted of mice actively immunized with *E. coli* transformed

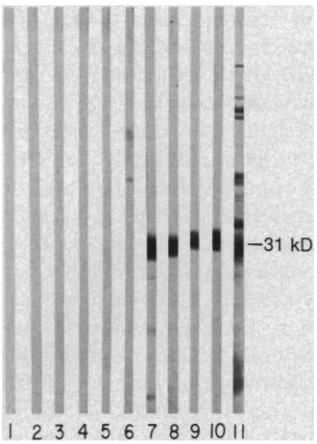

Fig. 1. Immunoblot of a protein extract of *B. burgdorferi* N40 stained with serum (diluted 1/100) from mice immunized with *E. coli* expressing OspA-N40, and *E. coli* lacking the OspA product. Column 1 is stained with normal mouse serum. Columns 2 to 6 (controls) are stained with serum from mice immunized with *E. coli* lacking the OspA product. Columns 7 to 10 are stained with serum from mice immunized with *E. coli* expressing OspA-N40. Column 11 is stained with anti *B. burgdorferi* strain N40 serum. The band at 31 kD represents OspA.

with pDC197-12. Mice actively immunized with *E. coli* expressing OspA were protected from infection, whereas the control animals immunized with *E. coli* that did not contain the OspA product readily developed infection. In addition the majority of the immunized animals were protected from clinical disease at 14 days (χ^2 $P \leq 0.05$) (Table 2).

To determine if the immune response to OspA could be enhanced to fully prevent the clinical manifestations of disease, mice were immunized with purified recombinant OspA-N40 expressed as a glutathione *S*-transferase fusion protein (*22*). Control mice were immunized with glutathione *S*-transferase. An extremely strong immune response to OspA was obtained since an antibody response could be detected to a dilution of 1:64,000 by immunoblot. Groups of five mice were challenged with *B. burgdorferi* N40 and sacrificed at 14 days. Histopathologic examination of the joints and heart in the animals immunized with purified OspA showed no evidence of disease, whereas the control animals readily developed arthritis and carditis (Table 2). Moreover, as expected, cultures of blood and spleen from OspA-immunized animals showed no evidence of infection, whereas all control mice were infected. Hence, the immune response that mice generate in response to recombinant OspA from *B. burgdorferi* N40 is sufficient to protect them from subsequent infection with *B. burgdorferi* N40. Studies at additional timepoints will determine the longevity of protection.

To test whether the protection is mediated by antibody, we recovered serum from mice immunized with OspA expressing *E. coli* and passively immunized mice with this serum. The mice were fully protected from infection at 5 days, suggesting that antibody to OspA generated in our actively immunized mice is sufficient for protection (Table 2). In addition an IgG3 (complement fixing) monoclonal antibody to OspA from *B. burgdorferi* N40 was protective in our assay, whereas an IgG1 (noncomplement fixing) was not. Indeed, chronic administration of a monoclonal antibody to OspA of *B. burgdorferi* strain ZS7 prevented *scid* mice from developing disease when challenged with *B. burgdorferi* ZS7 over a period of 21 days (*23*). The contribution of the cellular immune response in protection is not yet known.

It is important to determine whether the immune response to OspA is sufficient to protect animals from infection with different strains of *B. burgdorferi*. At the present, no taxonomic classification exists for the differentiation of various strains of *B. burgdorferi*. In general, isolates are classified as different on the basis of geographic location. It is surprising and encouraging that vaccination with OspA protects, because the previous passive immunization experiments with *B. burgdorferi* antiserum in the hamster model showed some degree of strain specificity, and OspA appears to be highly conserved among *B. burgdorferi* isolates. Since a comparison of the nucleotide sequence of OspA-N40, OspA-B31, and OspA-ZS7 showed a high degree of similarity, it is possible that protection should extend to different strains of *B. burgdorferi*. We therefore tested additional strains of *B. burgdorferi*, CD16 (*24*) and B31, and found that mice were protected from infection (Table 2). Further studies to determine if the protective effect of OspA is maintained with a wider variety of *B. burgdorferi* isolates will provide additional information on the breadth of protection.

Although *B. burgdorferi* infection in the mouse has similarities with human disease it is uncertain that vaccination with OspA will be effective in man. As antibodies to OspA appear late in human illness, suggesting that it is a poor immunogen, it must be shown that a strong immune response to recombinant OspA can be elicited in man. It is possible, however, that immunization of humans with recombinant OspA in an appropriate regimen and the use of adjuvant could give rise to a potent neutralizing antibody response. Studies in other animal models and man will extend the knowledge of the usefulness of OspA as a vaccine for Lyme borreliosis.

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strips. The strips were washed, incubated with a 1 5200 dilution of alkaline phosphatase–labeled goat antibodies to mouse IgG and developed with nitroblue tetrazolium 5-bromo 4-chloro-indolyl phosphate.

22. The OspA-N40 gene amplified by PCR was cloned into Eco RI and Bam HI sites of pGEX-2T (Pharmacia) in frame with the glutathione *S*-transferase gene The recombinant plasmid was used to transform *E. coli* strain JM109. Recombinant fusion protein production was induced with 1 mM IPTG. Cells were washed in PBS, suspended in 1/100 the volume PBS with 1% Triton and lysed by sonication. The lysate was centrifuged at 13,000 rpm and the supernatant was placed over a glutathione–Sepharose 4B column (Pharmacia). The glutathione *S*-transferase OspA-N40 fusion protein was eluted with 5 mM glutathione. Purified glutathione *S*-transferase was prepared in a similar fashion from *E coli* transformed with pGEX-2T.
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12 July 1990; accepted 19 September 1990

Probing Immunosuppressant Action with a Nonnatural Immunophilin Ligand

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The immunosuppressants FK506 and rapamycin bind to the same immunophilin, FK506 binding protein (FKBP), and inhibit distinct signal transduction pathways in T lymphocytes. A nonnatural immunophilin ligand, 506BD, which contains only the common structural elements of FK506 and rapamycin, was synthesized and found to be a high-affinity ligand of FKBP and a potent inhibitor of FKBP rotamase activity. Whereas 506BD does not interfere with T cell activation, it does block the immunosuppressive effects of both FK506 and rapamycin. Thus, the common immunophilin binding element of these immunosuppressants, which is responsible for rotamase inhibition, is fused to different effector elements, resulting in the inhibition of different signaling pathways. Inhibition of rotamase activity is an insufficient requirement for mediating these effects.

IN ADDITION TO THEIR THERAPEUTIC potential, the immunosuppressive agents cyclosporine A (CsA), FK506, and rapamycin are useful probes of the T cell activation process (Fig. 1) (*1–4*). CsA and FK506 inhibit a T cell receptor (TCR)–mediated signal transduction pathway that results in the transcription of early T cell activation genes, including interleukin-2 (IL-2). Despite the structural similarity to FK506, rapamycin has no effect on the production of IL-2, yet potently inhibits the response of the T cell to activation through the IL-2 receptor (*2*). Although they specifically inhibit different T cell activation events, rapamycin and FK506 have also been shown to inhibit each other's actions (*3, 4*).

These three immunosuppressants bind to proteins termed immunophilins with high affinity. CsA (K_d = 30 nM) is bound by human cyclophilin (*5*), and both FK506 (K_d = 0.4 nM) and rapamycin (K_d = 0.2 nM) are bound by human FK506 binding protein (FKBP) (*4, 6, 7*). Although unrelated in amino acid sequence (*8*), cyclophilins and FKBPs catalyze the interconversion of the cis- and trans-rotamers of the peptidyl-prolyl amide bond of peptide and protein substrates. These rotamase enzymes are inhibited by their respective immunosuppressant ligands; FK506 and rapamycin do not inhibit cyclophilin and CsA does not inhibit FKBP (*6–8*).

The ability of FK506 and rapamycin to inhibit each other's actions suggests a role for either a single immunophilin or separate immunophilins that share a common receptor site (hereafter referred to as the "common receptor site") in mediating the actions of these drugs (*4*). Rapamycin and FK506 appear to be mimics of a leucyl-prolyl twisted amide bond (*9, 10*), and may therefore mimic the action of an endogenous ligand that contains a peptidyl (perhaps leucyl)-prolyl dipeptide fragment as a structural motif for the recognition of an immunophilin regulatory site (the "common receptor site"). We explored the hypothesis that FK506 and rapamycin are comprised of a common immunophilin binding element that is fused to distinct effector elements that determine the T cell activation signaling pathway with which the drug will interfere.

We designed a molecule, termed 506BD, to adopt a geometry similar to that of the putative immunophilin binding domain found in FK506 (Fig. 1). To emulate the conformation of the cis-rotamer of FK506 [observed in the solid state by x-ray diffractometry (*11*)], a scaffolding element was installed to limit the conformational freedom of 506BD. 506BD lacks the putative effector elements of both rapamycin and FK506. Therefore, 506BD should not exhibit the T cell inhibitory properties that are characteristic of either FK506 or rapamycin, yet, by virtue of its anticipated immunophilin binding properties, 506BD should inhibit the actions of both FK506 and rapamycin.

The isopropyl substituent of 506BD was included to introduce a conformational constraint, involving the avoidance of a −gauche/+gauche (*syn*-pentane) local conformation, that is present in FK506. The trans-enoate spacer was chosen to fix the distance of the attachment sites in the same geometry observed in the solid-state conformation of FK506. We also synthesized a truncated 506BD that lacked the cyclohexyl moiety (des CyH-506BD) to assess its role.

An efficient synthetic pathway was developed that provided 506BD in useful quantities and in a stereochemically homogeneous form (Fig. 2). The asymmetric synthesis began with the six-carbon chain **1**, which had previously been prepared from mannitol in four steps (*12*). The pyranose **2** was obtained as a mixture of anomers from **1** in four steps; only the major β-anomer is shown. Stereoselective introduction of the allylic methoxyl group in **3** followed subjection of **2** to a one-pot reduction-Grignard addition sequence (*13*), whereas the benzyloxy bearing stereocenter in **4** was introduced by a stereoselective ketone reduction (*14*). After ring opening and homologation of **4** into **5**, the three dioxolane stereocenters of **6** were produced in a stereospecific fashion by an asymmetric osmylation reaction (*15*) and then acetal protection. The coupling partner **8** was prepared by metalation and hydroxyalkylation of the vinyl bromide **7**, which we have reported earlier in the course of a total synthesis of FK506 (*16*). The coupling of **6** and **8** was achieved by amide bond formation, while macrocyclization of the resultant **9** was accomplished by an intramolecular Horner-Emmons reaction (*17*) that produced **10** as a single E-olefin diastereomer. Following selective deprotection of the cyclic acetal and oxidation of the resultant diol to a diketone, a final deprotection reaction resulted in a transannular hem-

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