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Protective immunization with plasmid DNA containing the outer surface lipoprotein A gene of *Borrelia burgdorferi* is independent of an eukaryotic promoter

Plasmid DNA encoding the outer surface lipoprotein A (OspA) of *Borrelia burgdorferi* under the control of either strong eukaryotic/viral or its own bacterial promoter was injected intramuscularly (m. tibialis anterior) or intradermally into BALB/c and AKR/N mice. OspA-specific antibodies and OspA-reactive T helper 1 cells (Th1) were induced only with those plasmids containing the *ospA* structural gene including its own regulatory control region immediately upstream. In the absence of the *ospA* promoter, no or only marginal immune responses to OspA were obtained, even when strong eukaryotic promoter/enhancer elements were present. Together with the finding that the *ospA* promoter is active in a mouse B-lymphoma line, the data suggest that spirochetes are able to express at least part of their genes in the mammalian environment. Mice previously vaccinated with the relevant *ospA* plasmid DNA were protected against subsequent experimental challenge with a virulent strain of *B. burgdorferi*, as measured by the appearance of antibodies to a prominent protective epitope (LA-2) and the failure to re-isolate spirochetes from ear biopsies. In addition, C.B-17 severe-combined immunodeficient mice could be protected against infection by passive transfer of immune sera from *ospA* plasmid DNA-inoculated normal mice. Protective LA-2-related antibody titers obtained after repeated immunization persisted for 200 days and longer. This simple procedure of immunization using plasmid DNA consisting of a prokaryotic gene under the control of its own promoter holds great promise for the development of alternative subunit vaccines against bacterial infections, including Lyme disease. In addition, the availability of this novel prokaryotic promoter element now allows the study of the basis for the differential expression of bacterial genes in prokaryotic and eukaryotic environments.

1 Introduction

Lyme borreliosis is the leading vector-borne bacterial disease in Europe and USA [1]. The etiological agent, *Borrelia burgdorferi*, is primarily transmitted by the globally distributed ticks of the genus *Ixodes*. Dissemination of spirochetes through the blood and lymph occurs early after infection and may lead to a multisystem illness with manifestations in the skin, heart, musculoskeletal, and central nervous system [1]. Although patients can be successfully treated with antibiotics in the early stage of disease, it is unclear whether currently applied regimens always lead to the eradication of spirochetes [2]. Furthermore, spirochete-specific humoral and cellular immune responses generated during infection do not always protect patients against the development of disease [1].

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Abbreviations: **OspA:** Outer surface lipoprotein of *Borrelia burgdorferi* **pOspA:** Plasmid containing *OspA* **rlipOspA:** Recombinant OspA lipoprotein **i.m.:** Intramuscular **i.d.:** Intradermal **MDP-OspA:** Delipidated recombinant Met-Asp-Pro-OspA **SCID:** Severe-combined immunodeficient **IS:** Immune serum

Key words: Lyme disease / Vaccination /*Borrelia burgdorferi* / Spirochetes / Gene regulation

In search for a suitable polypeptide vaccine against Lyme disease, the outer surface lipoprotein A (OspA) has been found to be the most promising candidate in mice [3–5]. It was found that immunization of mice with recombinant OspA lipoprotein (rlipOspA) leads to the activation of B and T cells and that both lymphocyte populations are essential for the generation of optimal levels of protective antibodies [6]. However, because of the antigenic variation of OspA within the species of *B. burgdorferi* sensu lato [7, 8], the cross-protective potential of antibodies generated to one particular OspA genotype is still debated [9–11]. At present, rlipOspA, which has been shown to be safe, clinically well-tolerated, and immunogenic [12, 13], is being tested for its efficacy in phase III clinical trials. In spite of these achievements, the major drawback of this approach is the time-consuming and costly procedure inherent to the production of recombinant polypeptide vaccines.

Recent advances in molecular biology have led to the development of novel approaches for the induction of protective immune responses based on plasmid DNA [14–16]. Intramuscular (i.m.) and intradermal (i.d.) injections of plasmid DNA in mice have been demonstrated to result in the efficient uptake of DNA by cells of skeletal muscle and skin and the induction of cellular and humoral immune responses against the encoded protein [14–17]. Plasmids

were shown to be maintained in an episomal, circular form and do not replicate [14, 15, 18]. The expression of proteins, however, persisted in tissues of skeletal muscle and heart for several months [18, 19]. This novel technique, termed DNA-based vaccination, has been applied to viral [16, 20, 21], mycoplasma [22], and parasite antigens [23], and was shown to result in protection against infection.

We now show that DNA-based vaccination is also suitable to introduce outer surface proteins of *B. burgdorferi*, such as OspA, into the antigen processing pathway and to generate both protective humoral and cellular responses in mice. Most importantly, *ospA* gene expression was found to be independent of eukaryotic promoter/enhancer elements. The fact that bacterial genes can be independently activated in the eukaryotic environment may have import implications for pathogenesis and protection in the infected host.

2 Materials and methods

2.1 Animals and immunization protocol

Adult female BALB/c, AKR/N, and C.B-17.SCID mice (6 to 8 weeks old) were bred under specific pathogen-free conditions at the Max-Planck-Institut für Immunbiologie, Freiburg, Germany. BALB/c and AKR/N mice were repeatedly immunized by injection into tibialis anterior muscles (i.m.) or i.d. into the hind legs, with various doses of plasmid DNA containing *ospA* or control plasmids.

2.2 Bacteria and recombinant OspA lipoprotein

The virulent low-passage tick isolate *B. burgdorferi* ZS7 was grown in Barbour-Stoenner-Kelly medium and harvested as described [3]. rlipOspA and the delipidated derivative of lipOspA, Met.-Asp-Pro-OspA (MDP-OspA) were expressed in *Escherichia coli* according to previously described protocols. The recombinant protein preparations were purified by gel filtration (HR-4009), butanol extraction and Q-Sepharose chromatography [11, 24].

2.3 Construction and purification of expression vectors

Large-scale purification of expression vectors was conducted using the Qiagen Plasmid Mega Kit System (Qiagen GmbH, Hilden, Germany), according to the protocol of the manufacturer. Purified plasmids used in this study (Fig. 1a, b) were adjusted to a final DNA concentration of 1 mg/ml.

Plasmid pBosOspA contains the *ospA* gene from *B. burgdorferi* ZS7, including 140 bp of its immediately upstream control region (Fig. 1b) [25] cloned into the Bst XI site of pEF-Bos that contains the promoter/enhancer region of the human elongation factor 1α (EF-1α) gene (Fig. 1a) [26]. Plasmid pBosOspAr harbors the identical *ospA* gene fragment in the inverted orientation. The same *ospA* gene fragment was also cloned into the Bam HI site of plasmid pKEX-1 containing the promoter/enhancer region of the human cytomegalovirus (HCMV-IE) [27] and pBSIISK$^+$ (Stratagene, Heidelberg, Germany) to generate pKEX-OspA and pOspA, respectively. Plasmid pOspAcod was generated from pOspA by deleting the 140-bp DNA fragment containing the *ospA* upstream control region. pBosOspA17/48 was generated in a similar way with the exception that a 200-bp DNA fragment (bp -140 to bp $+60$) containing the *ospA* upstream control region and the nucleotide sequence encoding the 20 N-terminal amino acids of OspA was deleted [25, 28]. Plasmids were propagated in the *E. coli* strain DH5α (GIBCO BRL, Eggenstein, Germany). The nucleotide sequence of *ospA* has been submitted to the EMBL database with the accession number X16467.

2.4 Transfection and Northern blot analysis

The mouse B-lymphoma K46 [29] was propagated and transfected by electroporation as described [30] except that 20 μg of each plasmid was used. After 36 h, cells were washed twice in ice-old PBS and resuspended (1×10^7–2×10^7) in ice-cold TNE (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 10 mM NaCl). Total cytoplasmic RNA was extracted by lysing the cells in 0.1 % NP40, followed by centrifugation at 7600 × g for 5 min at 4 °C, followed by phenol extraction of the supernatant containing the RNA. Northern blot analysis of equal amounts of RNA from each transfection was performed by standard procedures [31] using a radiolabeled OspA probe (gel-isolated from Bam HI-digested pOspA). As a loading control, a mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA probe was used.

2.5 ELISA, mAb LA-2 competition ELISA and Western blot analysis

B. burgdorferi-specific antibodies (IgM, IgG, IgG1, IgG2a, IgG2b, and IgG3) were measured in a solid-phase ELISA as described [32], with the modification that plates were pre-coated with rlipOspA (1 μg/ml) instead of spirochete lysate.

Antibodies with the same specificity as the mAb LA-2, which was shown before to be protective in a mouse model for *B. burgdorferi* infection [3], were measured by a competition ELISA. Briefly, wells of ELISA microplates (Nunc Immunoplate Maxisorp, Rostilde, Denmark) were coated overnight at 4 °C with 100 μl rlipOspA (0.5 μg/ml in 0.05 M, Na_2CO_3/$NaHCO_3$, pH 9.6). After washing with PBS containing 0.05 % Tween 20 (PBS-T), the plates were incubated with 200 μl PBS/1 % BSA for 30 min at room temperature with shaking. Subsequently, wells were incubated for 2 h with 100 μl of serial twofold dilutions of mouse sera diluted in PBS/0.2 % BSA containing 0.05 % Tween 20. For the calibration curve, mouse serum was replaced by serial twofold dilutions of purified mAb LA-2 (highest concentration: 4 μg/ml). After washing, a limiting concentration of mAb LA-2, conjugated to peroxidase (in PBS/BSA 0.2 %, 0.05 % Tween 20; amount of peroxidase-labeled mAb LA-2 was adjusted to a concentration sufficient to reach the plateau level in a previous competition assay with rlipOspA) was added to each well. The determination of this critical concentration of peroxidase-labeled mAb LA-2 is important to achieve maximal sensi-

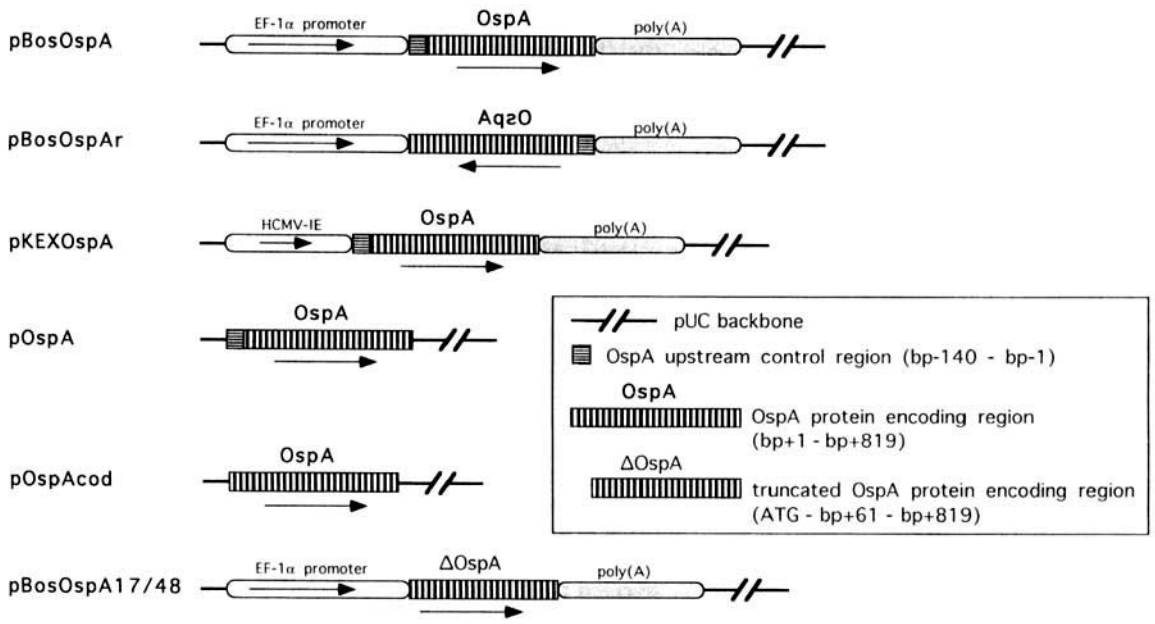
tivity of the assay. Plates were incubated for 30 min at room temperature with shaking. After washing, 100 µl substrate solution [4 mg o-phenylenediamine (Sigma P 8787, St. Louis, MO) in 10 ml 0.1 M citrate pH 4.5 + 5 µl 30% H_2O_2] was added to each well. After a 15-min incubation at room temperature in the dark, the reaction was stopped by addition of 50 µl 1 N HCl. The absorbance was read at 490 nm using an ELISA plate reader. The absorbance was converted in equivalents of mAb LA-2 using the calibration curve and the 4-parameter method.

For immunoblotting, spirochetes (strain ZS7) were lysed in SDS buffer, separated by SDS-PAGE on 12% polyacrylamide gels and transferred to nitrocellulose. Subsequently, individual nitrocellulose stripes were incubated with the respective sera (diluted 1 : 100 followed by goat anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b or IgG3 antibodies labeled with alkaline phosphatase as the second reagent as described [32].

2.6 T cell proliferation assay and test for cytokine production

Lymph node (a pool of axillary, inguinal and popliteal) or spleen cells were enriched for T cells by treatment for 2 h

a



b

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          14-mer                14-mer
AGAACCAAACTTAATTAAAACCAAACTTAATTGAAGTTATTATCATTT

                                -35          -10
TATTTTTTTTCAATTTTCTATTTGTTATTTGTTAATCTTATAATATAATTA
                      -35                -10

TACTTGTATTAAGTTATATTAATATAAAAGGAGAATATATTatg
+1                          RBS          OspA
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Figure 1. (a) *ospA* plasmid constructs used for DNA vaccination. The *B. burgdorferi* ZS7 *ospA* encoding gene (vertically hatched bars) including 140 bp (horizontally hatched bars) of its upstream control region was inserted in both orientations (sense = pBosOspA; antisense = pBosOspAr) into the Bst XI site of pEF-Bos that contains the promoter/enhancer region of the human EF-1α chromosomal gene [26]. Plasmid pKEXOspA contains the identical *ospA* gene fragment under the control of the HCMV-IE promoter/enhancer element cloned into the Bst XI site of pKEX-1 [27]. Similarly, the same *ospA* gene fragment was cloned into the Bam HI cloning site of pBSIISK$^+$ to generate pOspA. Plasmid pOspAcod is identical to pOspA except that the upstream control region of *ospA* was deleted. Plasmid pBosOspA17/48 is identical to pBosOspA, with the exception that a 200-bp deletion at the 5′ end of the *ospA* gene fragment (bp − 140 to bp + 60) was introduced and instead, an artifical ATG start codon was inserted. (b) Nucleotide sequence of the upstream control region of the *ospA* gene in *B. burgdorferi* ZS7. The respective − 35 (TTGTTA) and − 10 (TATAAT) regions of the promoter, ribosome binding sites (RBS) and the transcriptional start point (+ 1) are underlined. Direct repeats of the 14-base sequence (14-mer) are indicated by double underlines.

with magnetic beads coated with goat anti-mouse IgG $F(ab')_2$ (Paesel, Frankfurt, Germany). The resulting cell preparations were analyzed by FACS and consisted of >94% (spleen) or >98% (lymph node) T cells ($CD3^+$), and <3% (spleen) or <1% (lymph node) B cells (Ig^+).

Enriched T cells (2×10^5) from mice previously immunized twice with either pEF-Bos (control plasmid), pBos-OspA or pOspA were incubated with preparations of either sonicated *B. burgdorferi* organisms (20 μg protein/ml), lipOspA or MDP-OspA (2 μg/ml) in culture medium (Iscove's, 20% FCS) in a total volume of 200 μl in round-bottom MicroWell plates (Nunc). Production of cytokines was measured in culture supernatants obtained after 24 h. Proliferation was assessed in triplicate on day 3 by adding [^{3}H]thymidine for the last 12 h.

Supernatants were tested for IFN-γ and IL-4 using mAb pairs R4-6A2/XMG1.2 and 11B11/BVD4-1D11, respectively (PharMingen, San Diego; CA) in standard sandwich ELISA according to the manufacturer's instructions.

2.7 Challenge experiments and protection

2.7.1 Passive protection

SCID [H-2^d] mice were reconstituted with either pooled immune sera (IS) of OspA plasmid DNA (pOspA)-immunized BALB/c mice or mAb LA-2 [33] or PBS. The IS transferred were adjusted to contain 5 μg LA-2 equivalents (total/mouse). IS or mAb LA-2 (5 μg/mouse) were given i.p. and 1 h later, the recipients were challenged experimentally (s.c.) with 10^5 *B. burgdorferi* ZS7. Mice were monitored for the development of clinical arthritis under blinded conditions and for the presence of spirochetes by cultivating ear biopsies in BSK medium after 27 days as described [9, 34]. The development of clinical arthritis in the tibiotarsal joints was scored as described [35] using swelling and reddening as criteria: ++, severe; +, prominent; (+), moderate; +/−, mild; (+/−), mainly reddening without significant swelling; and −, no clinical signs of arthritis. At the end of the observation period (day 27), mice were killed and the joints and heart were fixed in 10% formaldehyde and embedded in paraffin wax. Tissue sections were scored in a blinded fashion for histological alterations as described [35].

2.7.2 Active protection

BALB/c mice previously immunized with either pBos-OspA, pOspA, pEF-Bos or left untreated, were experimentally challenged either once or repeatedly with 1×10^4 or 1×10^6 spirochetes (ZS7). Between 12 and 96 days post-infection (p.i.) mice were investigated for the presence of spirochetes by the cultivation of ear biopsies as described [32].

3 Results

3.1 Immunization of mice with plasmid DNA containing the OspA gene leads to the production of specific antibodies

BALB/c and AKR/N mice were repeatedly inoculated with the indicated plasmid DNA (Fig. 1a) in the quadriceps of both legs. OspA protein expression was demonstrated by analyzing the production of OspA-specific antibodies on rlipOspA by ELISA (Table 1) or by Western blot (Fig. 2). After the first immunization with pBosOspA, monospecific antibodies to OspA were already detectable in both mouse strains when tested on day 30 p.i. (Table 1, experiment 1; Fig. 2). In contrast, OspA-specific antibodies could not be detected in mice immunized with pEF-Bos (control plasmid). The amount of antibodies significantly increased with further immunizations, reaching plateau levels of more than 0.5 mg/ml in BALB/c and AKR/N mice after the second or third boost. The antibody response was dose-dependent, with higher amounts of DNA providing a faster immune response as well as higher antibody titers (Table 1, exp. 2). Similar antibody titers were also obtained from BALB/c mice following intradermal delivery of pBosOspA (Table 1, exp. 3). For comparison, pooled sera from mice infected with 10^8 spirochetes contained ~0.3 mg/ml OspA-specific antibodies; hyperimmune sera from rlipOspA-immunized mice contained ~4 mg/ml OspA-specific antibodies (Table 1). When eukaryotic and viral promotors, i.e. EF-1α (pEF-Bos) and HCMV-IE (pKEX-1), were compared for their efficacy to induce OspA-specific antibodies in BALB/c and AKR/N mice, the EF-1α promoter was more efficient (Table 1, exp. 4). This is in line with previous results and indicates that EF-1α has a higher potential than HCMV-IE to direct transcription and translation of *ospA* [26]. Surprisingly, immunization of mice with pBos-OspAr harboring the *ospA* gene in the inverse orientation also led to the generation of OspA-specific antibodies with kinetics and titers similar to those observed with pBos-OspA (Table 1, exp. 1). This indicated that the eukaryotic EF-1α promoter is not essential for high expression of OspA in mice. This assumption was verified by showing that the application of pOspA, which contains the *ospA* gene of *B. burgdorferi* ZS7 including its upstream control region but lacks any eukaryotic promoter sequences, also led to the production of OspA-specific antibodies in BALB/c mice (Table 1, exp. 5; representative of three independent experiments). Following the fourth immunization with pOspA, IS of these mice contained OspA-specific antibodies at levels (0.5 mg/ml) similar to those observed with pBosOspA. No or only marginal levels of OspA-specific antibodies were observed in mice immunized with either pOspAcod (Table 1, exp. 5) or pBosOspA17/48 (Table 1, exp. 1).

3.2 Transient transfection of target cells with plasmid DNA containing the OspA gene results in expression of specific mRNA

That the *ospA* promoter is also active in eukaryotic cells was directly tested by transiently transfecting several OspA expression plasmids into the mouse B-lymphoma K46, followed by Northern blot analysis of cytoplasmic RNA. Fig. 3 shows that the *ospA* promoter alone (lane 4)

Table 1. Development of OspA-specific antibodies in serum of BALB/c and AKR/N mice repeatedly immunized with plasmid DNA containing *ospA*[a)]

Exp.	Mouse strain	Plasmid or immunogen	Amount of anti-OspA (LA-2 equivalents*) antibodies (μg/ml) 1st	2nd	(LA-2)*	3rd	(LA-2)*	4th	(LA-2)*	Control IS
1	BALB/c	pEF-Bos	<0.1	<0.1	(<0.5)					
		pBosOspA	13	474	(137)	641	(71)			
		pBosOspAr	24	197	(75)	306	(52)			
		pBosOspA17/48	<0.1	<0.1	(<0.5)					
	AKR/N	pEF-Bos	<0.1	<0.1	(<0.5)					
		pBosOspA	nd	1100	(455)	534	(nd)			
		pBosOspAr	20	616	(nd)	583	(70)			
		pBosOspA17/48	<0.1	<0.1	(<0.5)					
2	BALB/c	pBosOspA								
		50 μg		15	(1.6)	224	(19)	733	(136)	
		5 μg		3.2	(7.4)	45	(4.1)			
		0.5 μg		1.4	(0.7)	15	(1.3)			
3	BALB/c	pBosOspA		282	(21.2)	926	(84)			
4	BALB/c	pBosOspA		75	(40)	150	(25)			
		pKEXOspA		10	(7.6)	105	(3.9)			
	AKR/N	pBosOspA		129	(103)	236	(62)			
		pKEXOspA		19	(7.6)	30	(4.8)			
5	BALB/c	pOspA		20	(1.8)	203	(15)	493	(31)	
		pOspAcod		<0.1		<0.1		<0.1		
Control	BALB/c	10^8 *B. burgdorferi* ZS7								308 (28)
	BALB/c	rlipOspA								4074 (1120)

a) BALB/c and AKR/N mice were immunized one to four times with the indicated plasmids (50 μg: exp. 1, 3, 4, 5; 50 μg, 5 μg, 0.5 μg: exp. 2). Sera were taken at the following intervals p.i.: Exp. 1: first, day 35 p.i.; second, day 15; third, day 15; Exp. 2: second, day 7; third, day 9; fourth, day 98; Exp. 3: second, day 8; third, day 9. Exp. 4: second, day 7; third, day 6; and Exp. 5: second, day 6; third, day 7; fourth, day 7. Pooled sera (five mice) were tested for OspA-specific antibodies by ELISA on rlilpOspA and for LA-2-related specificity in competitive ELISA as described in Sect. 2.5. BALB/c anti-10^8 *B. burgdorferi*. ZS7 IS is a hyperimmune serum pool derived from various bleedings taken between days 30 and 80 p.i.; BALB/c anti-rlipOspA IS is a hyperimmune serum pool derived from various bleedings taken between days 10–80 following the third immunization with rlipOspA (10 μg in ABM adjuvant, three times, biweekly intervals).

is several times stronger than the strong eukaryotic EF-1α promoter (lane 3) and that both promoters act synergistically when arranged in tandem on the same plasmid (lane 2). Transfection with a construct carrying neither the EF-12 promoter nor the *ospA* promoter (lane 5) shows, if at all, very weak expression. Notable features of the sequence upstream of the OspA protein-encoding region include two closely spaced repeats of the 14-base sequence AACCAAACTTAATT, as well as two direct repeats of the −35 (TTGTTA) and −10 (TATAAT) boxes (Fig. 1b).

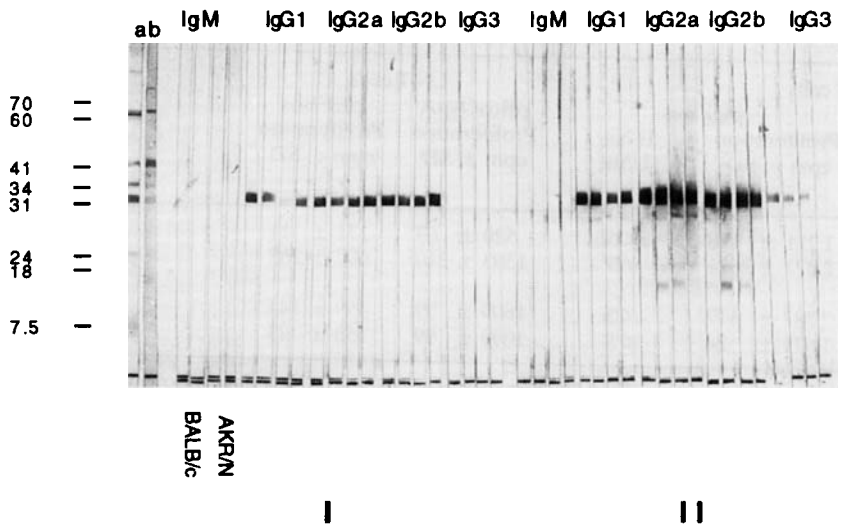

Figure 2. Isotypes (IgM, IgG1, IgG2a, IgG2b, and IgG3; four lanes each) of OspA-specific antibodies generated in BALB/c and AKR/N mice previously immunized (i.m.) once (I, day 0) or twice (II, day 35) with either pBosOspA (50 μg, lanes 1 and 3) or pBosOspAr (50 μg, lanes 2 and 4). Sera were taken at day 30 after the first and at day 10 following the second challenge (pool of five mice) and dilutions tested by Western blot analysis on ZS7 lysate. Lanes 1 and 2: BALB/c; lanes 3 and 4: AKR/N.

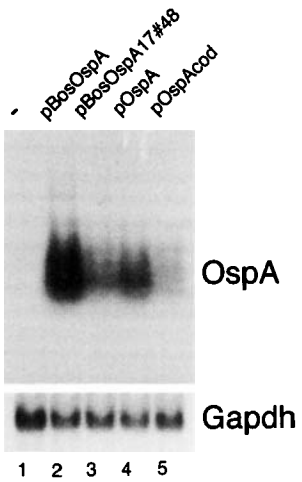

Figure 3. Northern blot analysis of OspA mRNA expression. The mouse B-lymphoma K46 was transiently transfected with the indicated plasmids and total cytoplasmic RNA was analyzed using a radiolabeled OspA probe (see Sect. 2.4). To control for equal loading of RNA, the blot was rehybridized with a probe for the ubiquitous mRNA encoding GAPDH.

3.3 Analysis of isotype patterns of anti-OspA antibodies and of OspA-specific T cells generated in response to plasmid DNA immunization

As shown in Fig. 2, IS from BALB/c and AKR/N mice immunized once (i.m.; Fig. 2 I) with pBosOspA contained antibodies of the isotypes IgG1, IgG2a and IgG2b and little, if any IgG3, when tested 30 days post infection; no OspA-specific IgM antibodies were detectable at this time point. After the second boost (i.m.; Fig. 2 II), IS of both mouse strains also contained OspA-specific antibodies of the IgG3 isotype. Similar isotype patterns were observed following immunization using the plasmid pOspA (data not shown).

The appearance of IgG1 and IgG3 antibodies to OspA in sera of mice following the first and second challenge, respectively, indicates that plasmid DNA immunization also leads to the induction of OspA-reactive T cells. To test this assumption, spleen and lymph nodes were removed from BALB/c and AKR/N mice after the boost with either pEF-Bos or pBosOspA. Enriched T cell populations (>90% pure) were restimulated *in vitro* with either *B. burgdorferi* ZS7 sonicate, rlipOspA or MDP-OspA or cultured in medium alone (Table 2). The proliferation rates of spleen and lymph node T cells previously sensitized *in vivo* to pBosOspA was approximately three and eight times higher, respectively, compared to control T cells when restimulated with MDP-OspA (Table 2, only shown for AKR/N mice). The differences in proliferative responses between naive and sensitized T cell populations were much less pronounced in the presence of *B. burgdorferi* sonicate or rlipOspA, an observation which is not unexpected in light of the recent findings that spirochete lipoproteins exhibit co-stimulatory activities for naive T cells [36, 37]. In addition, splenic T cells from pBosOspA- but not from pEF-Bos-inoculated AKR/N mice produced significant amounts of IFN-γ, but no IL-4 (data not shown) upon restimulation *in vitro* with MDP-OspA (Table 2). Similar results were obtained with T cells from pBosOspA-immunized BALB/c mice as well as with T cells from BALB/c and AKR/N mice previously sensitized to pOspA (data not shown).

3.4 Immunization of mice with plasmid DNA containing the OspA confers active and passive protection against challenge with *B. burgdorferi*

It has been shown that antibodies with specificity to the OspA epitope LA-2 [33] induced in hamsters [38], mice and dogs [39] could protect against challenge by infected ticks and/or by needle injection that as little as 0.3 μg mAb LA-2 was sufficient to protect hamsters. In addition, it was found that sera from monkeys and human volunteers immunized with rlipOspA can confer protection against experimental infection; in that case, 1 μg/ml LA-2-specific antibody was sufficient to achieve protection (Hauser and Voet, unpublished observation). We therefore tested IS from mice previously immunized with the plasmids shown in Fig. 1a for the presence of LA-2-related antibody specificities. As seen in Table 1, with no exception, all DNA vaccination protocols that resulted in the generation of OspA-specific antibodies also led to the induction of those related to the LA-2 epitope. After the third immunization,

Table 2. Induction *in vivo* of OspA specific T cells in mice immunized with plasmid DNA containing *OspA*[a)]

	Spleen T cells				LN T cells	
	pBosOspA		pEFBos		pBosOspA	pEFBos
Addition to culture	Proliferation cpm ± SD	IFN-γ U/ml	Proliferation cpm ± SD	IFN-γ U/ml	Proliferation cpm ± SD	Proliferation cpm ± SD
–	3632 ± 222	6.5	1211 ± 178	<5	520 ± 63	171 ± 11
B. burgdorferi sonicate	26128 ± 2899	11	12077 ± 2511	<5	1303 ± 210	607 ± 262
Lip-OspA	26355 ± 2505	16	7787 ± 617	<5	1609 ± 99	459 ± 144
MDP-OspA	9777 ± 91	30	1671 ± 24	<5	1099 ± 99	153 ± 23

a) AKR/N mice were immunized twice at day 0 and day 14 with either pBosOspA or pEF-Bos (50 μg). Seven days following the second challenge, spleen and lymph node (a pool of axillary, inguinal, politeal) cell populations from two mice were enriched for T cells: spleen (pBosOspA: 91.8% $CD3^+$, 4.8% Ig^+; pEF-Bos: 94.5% $CD3^+$, 2.4% Ig^+); lymph node (pBosOspA: 96.6% $CD3^+$, 0.3% Ig^+; pEF-Bos: 98.2% $CD3^+$, 0.7% Ig^+). T cell populations were incubated with either *B. burgdorferi* ZS7 sonicate (20 μg/ml), Lip-OspA (2 μg/ml) or MDP-OspA (2 μg/ml) *in vitro* and tested for proliferation and the production of IFN-γ and IL-4 (data not shown) as described in Sect. 2.6.

Table 3. Development of clinical arthritis and infection in C.B-17 SCID mice pre-treated with either immune serum to pOspA or mAb LA-2 and subsequently challenged with *B. burgdorferi* ZS7[a)]

Amount of serum or mAb transferred	Mouse no.	Clinical arthritis at day p.i. 7	9	16	20	27	Recultivation of spirochetes
PBS	1	–	–	+	++	++	+
	2	–	(±)	(+)	+	++	+
	3	–	–	++	++	++	+
Anti-pOspA (LA-2 equiv. 5 μg)	1	–	–	–	–	–	–
	2	–	(±)	–	–	–	–
	3	–	(±)	–	–	–	–
	4	–	–	–	–	–	–
mAb LA-2 (5 μg)	1	–	(±)	–	(±)	–	–
	2	–	–	–	–	–	–
	3	–	–	–	–	–	–

a) C.B-17. SCID mice were treated with the indicated amounts of anti-pOspA immune serum (adjusted for 5 μg LA-2 equivalents) or mAb LA-2 or with PBS and challenged 1 h later with 10^5 *B. burgdorferi* ZS7 spirochetes. Clinical arthritis and recultivation of spirochetes was monitored as described in Sect. 2.7.

Table 4. Protection of mice vaccinated with *ospA*-plasmid DNA against experimental challenge with *B. burgdorferi*[a)]

Mouse strain	Vaccinated with:	Challenge	Culture day p.i.	No. positive/ No. examined
BALB/c	none	1×10^4	12	0/5
		1×10^4	22	1/4
		1×10^6	60	2/3
			98	2/3
BALB/c	pEF-Bos	1×10^6	55	1/3
			93	1/3
BALB/c	pBosOspA	1×10^4	12	0/5
		1×10^4	22	0/5
		1×10^6	60	0/5
BALB/c	pOspA	1×10^6	55	0/3
			93	0/3

a) BALB/c mice were immunized three times with the indicated plasmid (50 μg). Fourteen days after the last challenge, mice were infected either once (1×10^4 or 1×10^6) or repeatedly with 10^4 (day 30 p.i) or 10^6 (day 60 p.i) ZS7 spirochetes. Infection was monitored by recultivation of spirochetes from ear biopsies at the indicated time, as described in Sect. 2.7.

most of the IS contained more than 10% LA-2-specific antibodies, a level similar to that obtained after infection with 10^8 spirochetes (Table 1). Protective titers of LA-2-specific antibodies were observed for more than 200 days following the third immunization with either pBosOspA or pOspA, even when only 0.5 μg plasmid DNA was applied (data not shown). For comparison, hyperimmune sera of BALB/c mice immunized with rlipOspA consisted of 30% LA-2-specific antibodies (Table 1).

To test for the protective capacity of OspA-specific antibodies induced by plasmid DNA vaccination, SCID mice were treated with IS from pOspA-inoculated mice (adjusted to contain 5 μg LA-2-equivalents) or with mAb LA-2 (5 μg, total) and subsequently challenged with 10^5 *B. burgdorferi* organisms (Table 3). Development of clinical arthritis (reddening and swelling) and recovery of spirochetes from ear biopsies were monitored. Inoculated, PBS-treated control SCID mice developed clinical arthritis starting between 9 and 16 days post challenge and resulted in severe swelling of the tibiotarsal joints between days 20 and 27. Spirochetes could be recultivated from all control mice. In contrast, SCID mice injected either with IS from pOspA-inoculated mice or with mAb LA-2 did not develop any significant signs of clinical arthritis under similar conditions and spirochetes could not be recultivated from ear biopsies (Table 3). The macroscopic findings were confirmed by histological examination (data not shown).

To evaluate the protection in actively immunized animals, BALB/c mice were injected twice with pBosOspA, pOspA, or pEF-Bos (control plasmid). Mice were subsequently infected either once or repeatedly with doses of 10^4 or 10^6 spirochetes ZS7 (s.c.) and monitored by re-isolation of spirochetes from ear biopsies. Nonvaccinated mice served as controls. As shown in Table 4, spirochetes could not be re-isolated from any of the mice vaccinated with pBosOspA or pOspA; in contrast, spirochetes were recovered, though to various extents, from untreated mice or mice vaccinated with the control plasmid DNA.

4 Discussion

4.1 Plasmid vaccines

In the present study, we demonstrate a new form of gene expression of the outer surface protein OspA of *B. burgdorferi*, the cause of Lyme disease, in the mammalian environment that has not been observed before. Although recent publications on DNA-based immunization suggested that strong eukaryotic promoters are necessary for gene expression in mammals [19, 26, 40], we now show by comparative analysis that the application of plasmid DNA containing the prokaryotic *ospA* gene including its own upstream regulatory region into mouse muscle or skin tissue leads to protective immunity similar to that achieved by immunization with the respective recombinant OspA lipoprotein. This approach to evoke an immune response against prokaryotic antigens by plasmid DNA immunization may be of more general applicability for the generation of serological reagents to those bacterial genes harboring promoter regions recognized by eukaryotic transcription factors. Moreover, the promoter of the bacterial *ospA* gene offers a new strategy for the development of powerful mammalian/bacterial shuttle vectors.

4.2 The prokaryotic *ospA gene* is expressed in an eukaryotic environment

It is unclear how the prokaryotic *ospA* gene is activated in the mammalian host. In light of recent observations, it is rather unlikely that plasmid DNA is randomly integrated into the host cell genome and thus subject to the control of eukaryotic promoter elements [18, 19]. In fact, we have shown by transient transfection into the mouse B-lymphoma line K46 that transcription from pOspA is stronger than from pOspA17/48. Assuming that the stability of the transcripts is similar, this indicates that even in eukaryotic cells, the upstream bacterial control region of *ospA* (140 bp) is a strong promoter able to drive the expression of the OspA.

At present, it is unknown which part of the *ospA* promoter mediates transcription. The similarity in structure between linear plasmids of *B. burgdorferi*, which encode several surface antigens, such as OspA, B, D, E, F and pG [34] and some viruses, suggests a eukaryotic/viral origin of these linear plasmids and a possible conservation of transcriptional regulatory elements between prokaryotic and eukaryotic systems [41]. Comparison of many regulatory elements in the promoters of eukaryotic, bacterial, and viral genes has revealed an important element, known as the TATA box in eukaryotes, which bears some resemblance to the Pribnow box in prokaryotic promoters [42, 43]. The *ospA* upstream control region, like those from several other *B. burgdorferi* surface antigens, is very A/T-rich, and TATA-like motifs can be found [25, 34]. Thus, these elements may be functional both in bacteria and eukaryotic cells. In addition, bacterial cAMP-responsive elements have been shown to be functional in eukaryotic cells [44]. A more detailed analysis of the *ospA* promoter is required to identify the *cis*-acting element(s) operative in the regulation of transcription.

Our findings have several important implications. It is tempting to speculate that bacteria have evolved strategies to express at least parts of their genome in mammalian cells to escape host defense mechanisms. In light of recent observations that foreign genes can be most effectively expressed in mammalian cells when associated with circular double-stranded plasmid DNA [14, 15, 18, 19], it may be of importance to note that the OspA protein is encoded by a gene located on a linear plasmid with covalently closed ends [41]. Thus, it is possible that those genes of *B. burgdorferi* which are encoded by plasmids, including OspA and other outer surface lipoproteins, are expressed in the eukaryotic environment.

For *B. burgdorferi*, one could envisage a situation in which antigens from spirochetes, including OspA, are introduced into the antigen-processing pathway by expression from respective linear/circular plasmid DNA taken up during phagocytosis by host cells. This could result in MHC class I expression of OspA-derived peptides. The destruction of these spirochete-infected target cells by specific cytolytic T cells may serve to weaken the phagocytic clearance of the spirochetes and lead to the liberation of still intact, surviving *B. burgdorferi* organisms which could either infect other cells or migrate to immuno-privileged sites. This hypothetical scenario is supported, at least partially, by previous findings indicating that spirochetes survive intracellularly [45, 46] and that they persist even in the presence of optimal protective antibody responses [47].

We can only speculate whether OspA protein is expressed by muscle cells or professional APC resident in or circulating through the muscle. It is clear from previous studies that injection of plasmid DNA leads to the expression of the encoded protein intracellularly and in the circulation [15, 18, 19]. Our data show that OspA is expressed in amounts sufficient to induce efficient humoral and cellular immune responses. Most probably, the OspA protein is expressed in its nonlipidated form, since the lipid moiety of bacterial proteins is unique to prokaryotic organisms [48]. Whether this is of relevance for the quality or magnitude of the immune response generated must await further detailed experimentation. The fact that no protective immunity was observed following treatment with pBosOspA17/48, despite its apparent ability to be transcribed (Fig. 3), may be due to the fact that the OspA protein produced from this construct lacks the N-terminal leader sequence. This sequence may be important for guiding OspA to a cellular compartment involved in evoking an immune response.

4.3 Vaccination of mice with plasmid DNA containing the OspA gene leads to protection against infection with *B. burgdorferi*

The specific B and T cell responses generated in BALB/c and AKR/N mice after immunization with plasmids containing the *ospA* gene including its own upstream control region are similar to those observed after challenge with rlipOspA [5, 6]. This is true with respect to the generation of OspA-specific antibodies, including their isotypes (Fig. 2 [32]). Moreover, plasmid DNA-vaccinated mice were readily protected against infection. In addition, adoptive transfer of IS from these mice led to full protec-

tion of recipient SCID mice upon challenge with spirochetes. The protective capacity of transferred immune sera was similar to that obtained following rlipOspA immunization as indicated by comparable amounts of LA-2 equivalent antibodies which have recently been shown to correlate positively with protection [38, 39].

Plasmid DNA vaccination of AKR/N and BALB/c mice also leads to activation of OspA-reactive T cells. The finding that supernatants of T cells restimulated *in vitro* only contained IFN-γ but no IL-4 suggests that this immunization protocol results in a preferential recruitment of Th1 cells. Although in line with previous studies in the human system [49], the data are in contrast to those of recent reports in the mouse [50, 51]. Following infection with *B. burgdorferi*, both IFN-γ and IL-4 was detectable in lymphocyte cultures upon restimulation with borrelial sonicate *in vitro:* C3H/HeJ T cells produced high levels of IFN-γ and low levels of IL-4, whereas the contrary was true for BALB/c T cells. However, it should be noted that in spite of the polarized cytokine patterns observed in the two mouse strains, the production *in vitro* of IL-4 by the respective T cell populations is always orders of magnitudes lower compared to IFN-γ when incubated with either spirochetal or polyclonal stimuli [50–52]. Thus, at present it is not known whether the lack of IL-4 production in our study is due to the distinct priming protocol or to a lower sensitivity of the cytokine assay system used.

Our data do not reveal whether plasmid DNA vaccination leads to the activation of both $CD4^+$ and $CD8^+$ T cells. However, preliminary experiments showed that immunization of mice with pOspA results in the generation of T cells reactive to MHC class-II-specific peptides [52]. This is of importance in light of the finding that the development of optimal protective antibody responses to OspA is dependent on T helper cells [6].

No side effects have been observed in mice vaccinated with either of the plasmid DNA applied in this study. Together with recent results showing that mice tolerant to OspA develop arthritis similar to nontransgenic littermates [53], the present data argue against this lipoprotein being a critical factor in the development of arthritis, as was recently suggested in the human system [54, 55].

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