

Resolution of experimental and tick-borne *Borrelia burgdorferi* infection in mice by passive, but not active immunization using recombinant OspC

Weimin Zhong¹, Lise Gern², Thomas Stehle¹, Crisan Museteanu¹, Michael Kramer³, Reinhard Wallich³ and Markus M. Simon¹

¹ Max-Planck-Institut für Immunbiologie, Freiburg, Germany

² Institut de Zoologie, Université de Neuchâtel, Neuchâtel, Switzerland

³ Institut für Immunologie der Universität Heidelberg, Heidelberg, Germany

Vaccination with outer surface protein A (OspA) of *Borrelia burgdorferi* prevents subsequent infection and disease in both laboratory animals and humans with high efficacy. OspA-based immunity, however, does not affect established infection due to the loss of OspA expression in the vertebrate host. We show here that repeated passive transfer of mouse and/or rabbit immune sera to recombinant GST-OspC fusion protein resulted in a dose-dependent resolution (1) of fully established arthritis and carditis as well as infection in needle-challenged C.B-17 SCID and (2) of infection in both experimentally and tick-infected BALB/c mice. Unexpectedly, active immunization of disease-susceptible AKR/N mice with GST-OspC only led to prevention but not resolution of disease and infection, in spite of high serum titers of OspC-specific Ab and the expression of *ospC* in tissue-derived spirochetes. The data suggest that the efficacy of OspC antibody-mediated immunity depends on the immunological history of the recipient and/or environment-dependent regulation of OspC surface expression by spirochetes *in vivo*. The results encourage further attempts to develop therapeutic vaccination protocols against Lyme disease.

Key words: Lyme disease / Therapeutic vaccination / Outer surface protein C / Original antigenic sin

1 Introduction

Lyme borreliosis is a frequent vector-borne infection caused by the spirochete *Borrelia burgdorferi* [1]. One of the puzzles of this multisystemic disease is the failure of men and mice to clear infection in many cases, despite pronounced humoral and cellular immunity to the infecting agent [1–4]. This is even more compelling since immune sera (IS) from experimentally and naturally infected mice are able to protect naive recipients from infection [5–7]. Further studies revealed that protective immunity against infection is mediated by Ab to various outer surface proteins (Osp) of *B. burgdorferi*, including OspA, B and C, and can be achieved by passive and active immunization [5, 8–11].

[1 18963]

Abbreviations: **GST-OspC:** Recombinant glutathione S-transferase-OspC fusion protein **rOspC:** Recombinant lipid-free OspC derivative **rLip-OspA:** Recombinant outer surface lipoprotein A **NRS:** Normal rabbit serum **IS:** Immune serum **p.i.:** Postinfection **RT:** Reverse transcription

Of the Osp known so far, OspA has been the most promising vaccine candidate against Lyme disease [12]. This is emphasized by the recent phase III clinical trials with over 10 000 individuals in the United States, which revealed a nearly 80 % protection of recipients against infection [13, 14]. However, the OspA-based vaccine is only applicable for prevention but not treatment of established infection [5, 15, 16]. The reason for that is unclear, but probably due to the fact that OspA is mainly expressed by spirochetes within ticks, and down-regulated upon their transmission to mammalian hosts [17, 18].

In contrast to OspA, OspC appears to be expressed in spirochetes, at least temporarily, in the vector [17] and the vertebrate host [18]. OspC is also immunogenic, because patients with Lyme disease as well as naturally or experimentally (low dose: $\leq 10^3$ spirochetes) infected mice readily produce Ab to OspC, but if at all, only occasionally to OspA [1, 6, 7]. Active immunization of gerbils and laboratory mice with recombinant OspC leads to their complete protection against experimental challenge and/or tick-borne infection with homologous *B. burgdorferi* isolates [11, 19, 20].

We have recently found a close correlation between high titers of serum anti-OspC Ab and resolution of infection in mice [16]. This suggests that, in principle, spirochetes are accessible and susceptible to OspC-specific Ab within affected tissues. Subsequent studies showed that passive transfer of OspC-specific mouse IS into experimentally infected SCID mice leads to protection as well as resolution of chronic arthritis and carditis, and clearance from infective spirochetes [16]. However, a similar study with contrasting results indicates that the protective capacity of OspC-specific Ab seems to critically depend on the nature of the immunogen used [21].

We have now studied in more detail therapeutic vaccination against established *B. burgdorferi* infection using OspC, and have analyzed the tissue localization of spirochetes and mRNA expression of *OspC* during infection.

2 Results

2.1 Protective potential of mouse and rabbit immune sera to OspC

In light of previous conflicting results [16, 21], we have re-examined two preparations of OspC from strain ZS7, i.e. the recombinant glutathione S-transferase-OspC fusion protein (GST-OspC) and its rOspC (lipid free) por-

tion for their immunogenicity and ability to induce protective Ab in BALB/c mice. With both protocols, animals seroconverted; pooled IS from mice repeatedly challenged with GST-OspC (3×) or rOspC (4×) contained 167 µg/ml and 66 µg/ml OspC-specific Ab, respectively. As shown in Table 1, passive transfer of 30 and 3 µg, but not of 0.3 µg of GST-OspC-specific or 3 µg of rOspC-specific Ab into C.B-17 SCID mice 1 h before challenge led to complete protection against disease and infection in all 12 mice in three independent experiments. In addition, the state of infection was monitored by recultivating of spirochetes from ear skin biopsies, a target organ consistently infected in laboratory mice [3, 22] and in reservoir hosts [23]. Furthermore, mouse IS to GST-OspC – and, as shown before [5, 16], to recombinant outer surface lipoprotein A (rLip-OspA) – completely prevented development of clinical arthritis and infection in all 5 C.B-17 SCID mice tested upon tick challenge (Table 2; [9, 10, 16]). As shown before, IS to rLip-OspA had a similar protective potential.

In essence, similar results were obtained with rabbit IS to GST-OspC, which, however, showed lower levels of protective potential (Table 1, Exp. 4). Repeated administration of either 360 µg (4×) or 1800 µg (4×) Ab to OspC starting at day 30 postinfection (p.i.), resulted in partial or nearly complete resolution, respectively, of fully established clinical arthritis (data not shown) and carditis

Table 1. Prevention of experimental *B. burgdorferi* infection in C.B-17 SCID mice by passive transfer of OspC-specific IS^{a)}

Experiment	Source of immune serum	Treatment	Dose	Clinical arthritis	Ear culture ^{b)}
1	BALB/c	–	–	3/3	3/3
		anti-GST-OspC	30 µg	0/3	0/3
			3 µg	0/3	0/3
			0.3 µg	1/3	1/3
2	BALB/c	–	–	3/3	3/3
		anti-GST	50 µl, undiluted	3/3	3/3
		anti-GST-OspC	3 µg	0/3	0/3
3	BALB/c	–	–	3/3	3/3
		anti-GST-OspC	3 µg	0/3	0/2
		anti-rOspC	3 µg	3/3	3/3
4	Rabbit	–	–	3/3	3/3
		anti-GST-OspC	30 µg	0/3	0/3
			3 µg	2/3	2/3
			0.3 µg	3/3	3/3

a) Pooled mouse or rabbit IS generated to GST-OspC or rOspC were passively transferred at indicated doses into C.B-17 SCID mice 1 h before experimental s.c. infection with 10^3 *B. burgdorferi* strain ZS7. Control mice received mouse IS specific for GST or remained untreated.

b) Spirochete re-cultivation from ear biopsies was done on day 32 p.i. for experiments 1, 2 and 3 and on day 21 p.i. for experiment 4, respectively.

Table 2. Prevention of tick-borne *B. burgdorferi* infection of C.B-17 SCID mice by passive transfer of GST-OspC- or rLip-OspA-specific IS^{a)}

Immune serum	Clinical arthritis (days p.i.)		Ear culture (days p.i.)	
	68	100	40	100
None	2/2	2/2	2/2	2/2
Anti-rLip-OspA	0/4	0/4	0/4	ND
Anti-GST-OspC	0/5	0/5	0/5	ND

a) C.B-17 SCID mice were treated with GST-OspC- or rLip-OspA-specific IS (10 µg/mouse) and challenged 1 h later with previously infected (*B. burgdorferi*, strain ZS7) nymphal ticks. Control mice remained untreated.

(Table 3 and Fig. 1). Histopathological analysis of heart tissue of infected mice showed that the massive inflammatory lesions, which were mainly associated with the valvulae regions but much less pronounced within the myocardium, were partially or completely resolved, when tested with either low (360 µg; 4x) or high (1800 µg, 4x) amounts of rabbit IS, respectively (Fig. 1). Again, as shown before with OspC-specific mouse IS [16], C.B-17 SCID mice treated with IS to GST-OspC (30 µg Ab) 1 h prior to experimental challenge with spirochetes did not develop valvulitis or myocarditis. Furthermore, after treatment of infected C.B-17 SCID mice with 360 µg/ml of OspC-specific rabbit Ab, spirochetes were not detectable at day 73 p.i. (day 44 after last treatment) but reappeared at day 115 p.i. (day 86 after last treatment; Table 3). In contrast, when treated with fivefold higher concentration of the same rabbit IS (1800 µg Ab), spirochetes could not be recovered from ear biopsy at either of the two time points (Table 3).

Table 3. Prevention and resolution of experimental *B. burgdorferi* infection in C.B-17 SCID mice by passive transfer of OspC-specific rabbit IS^{a)}

Rabbit immune serum (µg/mouse)	Interval of treatment (days p.i.)	Mouse	Clinical arthritis (days p.i.) ^{b)}				Histopathological examination of heart ^{c)}	Ear culture (days p.i.)					
			30	87	115	155		day ^{d)}	pericard.	myocard.	endocard.	30	73
Anti-GST-OspC (30 µg)	-1 h	1	-/-	-/-			88	-	-	-	-	-	ND
		2	-/-	-/-	-/-	-/-	155	-	-	-	-	-	ND
		3	-/-	-/-	-/-	-/-	155	-	-	-	-	-	ND
NRS	30, 35, 39, 44	1	+/+/+	+/+/+			88(44)	++	++	+	+	+	ND
		2	+/+/+	+/+/+	+/+/+	+/+	155(111)	++	++	+	+	+	ND
		3	+/+/+	+/+/+	+/+/+	+/+	155(111)	++	++	+	+	+	ND
Anti-GST-OspC (360 µg)	30, 35, 39, 44	1	+/+/+	±/±			88(44)	-	-	-	+	-	ND
		2	+/+/+	±/±	(±)/(±)	±/+	155(111)	+	+	-	+	-	+
		3	+/+/+	±/(±)	+/+/+	+/+	155(111)	++	+	-	+	-	+
Anti-GST-OspC (1800 µg)	30, 35, 39, 44	1	+/+/+	(±)/±			88(44)	±	-	-	+	-	ND
		2	+/+/+	±/(±)	(±)/±	(±)/(±)	155(111)	-	-	-	+	-	-
		3	+/+/+	±/±	±/(±)	+/±	155(111)	-	-	-	+	-	-

a) C.B-17 SCID mice were treated with the indicated doses of rabbit anti-GST-OspC IS either before (-1 h) or starting at day 30 p.i. In the latter case, control mice received normal rabbit serum (NRS). Recipients were infected s.c. with 10^3 *B. burgdorferi* strain ZS7.

b) Scoring of clinical arthritis in the right and left tibiotarsal joints: ++, severe; +, moderately severe; ±, mild swelling; (±), redding; -, no clinical signs.

c) Scoring for histopathological examinations of heart: ++, extensive; +, severe; ±, moderate; -, no lesion.

d) Day of histopathological examination p.i. (days after last Ab treatment).

2.2 Potential of anti-GST-OspC IS to prevent or resolve experimental and tick-borne infection in BALB/c mice

BALB/c mice were given GST-OspC-specific IS either before (-1 h; 10 μ g Ab, 1 \times) or repeatedly (30 μ g Ab, 4 \times in 3-day intervals) after experimental challenge, starting at either day 10, 20 or 40 p.i. (Fig. 2A) and the state of infec-

tion was determined by recultivation of spirochetes from ear biopsy specimens. Passive transfer of GST-OspC-specific IS (10 μ g Ab) prior to challenge completely protected all five mice against infection (Table 4). Most notably, sterile immunity was also achieved in all but two (18/20) infected BALB/c mice, independent of whether treatment with GST-OspC-specific IS started on either day 10, 20 or 40 p.i. In contrast, spirochetes were re-

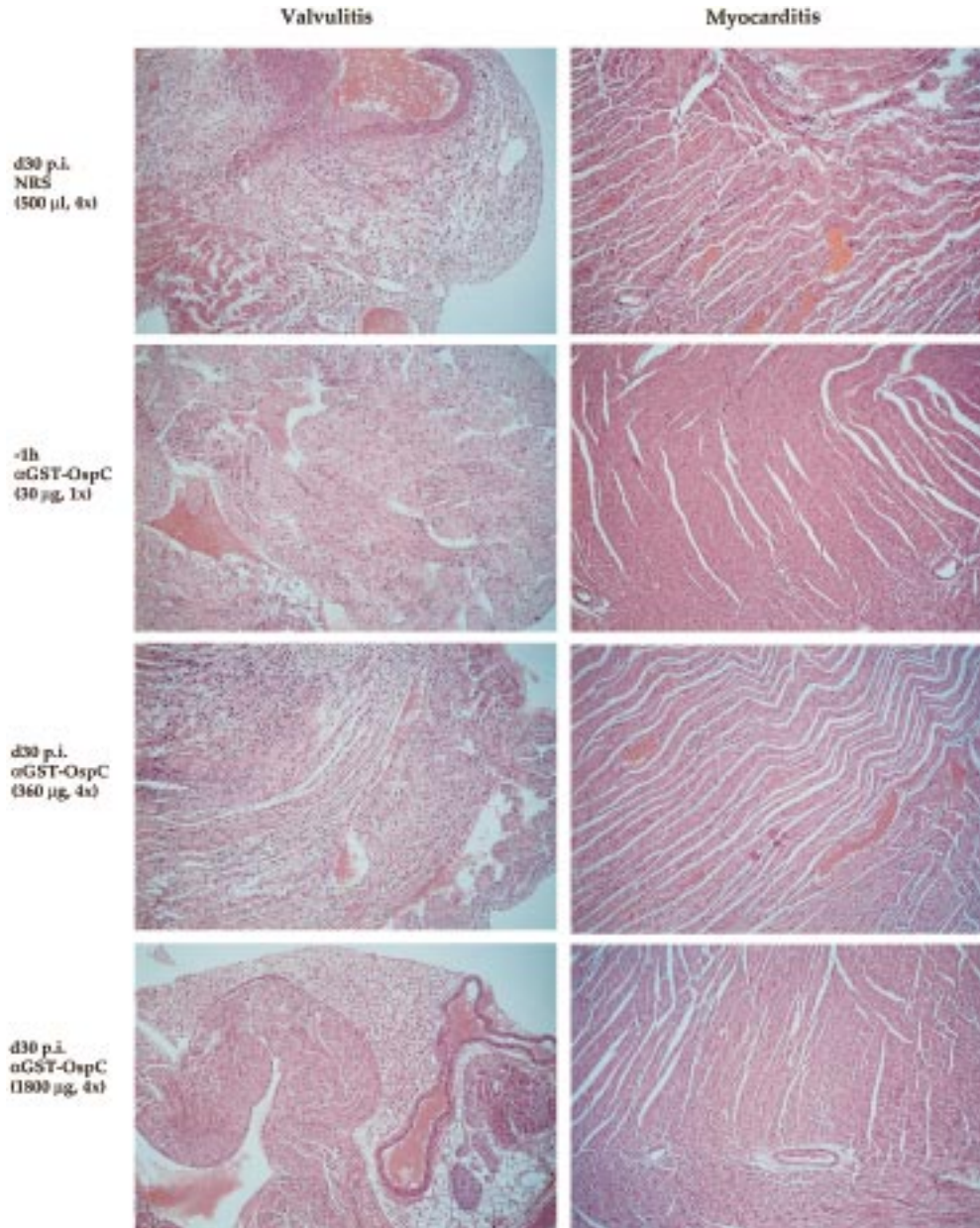


Figure 1. Histopathological examination of heart (day 155 p.i.) from *B. burgdorferi*-infected C.B-17 mice following either prophylactic or therapeutic treatment with rabbit IS specific for GST-OspC. One set of representative sections is shown out of four sections with similar results, taken at different sites of each paraffin-embedded heart sample.

isolated from four of five untreated, but infected mice when examined between days 42–58 and days 65–73 p.i., respectively. Similar results were obtained with BALB/c mice previously challenged by tick infection (Table 4).

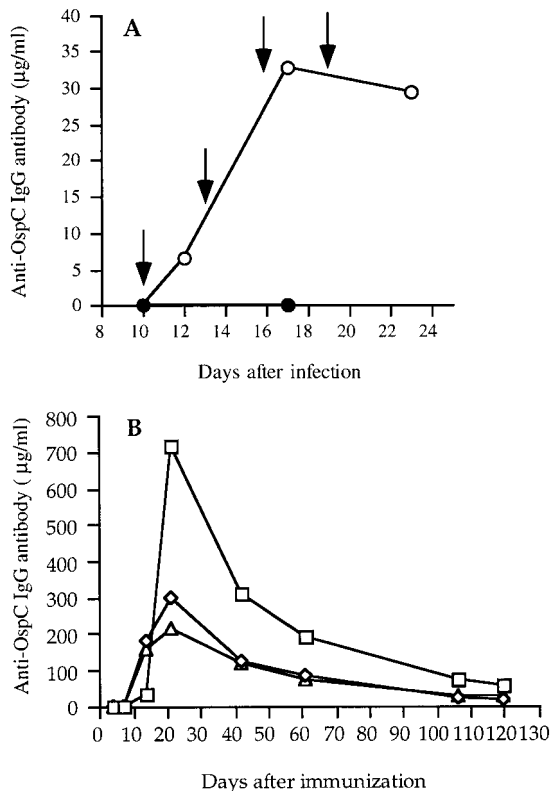


Figure 2. Kinetics of serum OspC-specific IgG Ab after passive (A) or active (B) immunization with GST-OspC. (A) BALB/c mice were infected s.c. with 10^3 ZS7 spirochetes. Ten days later the animals were injected with syngenic IS specific for GST-OspC ($30 \mu\text{g}$ Ab/mouse, i.p., 4 \times , 3-day intervals; open circles). Control mice remained untreated (closed circles). The arrows indicate the time of serum administration. Pooled sera (five mice/group) were taken at indicated times and the levels of OspC-specific IgG Ab were determined by ELISA. (B) Naive AKR/N mice were immunized with GST-OspC in adjuvant ($10 \mu\text{g}$ Ab/mouse, s.c., 3 \times , 7-day intervals). Serum samples from three individual mice (open squares, triangles and diamonds, respectively) were collected at indicated time intervals and the levels of OspC-specific Ab were determined by OspC-specific ELISA.

2.3 Effect of active immunization with GST-OspC on prevention and resolution of *B. burgdorferi*-induced disease and infection in AKR/N mice

We next tested whether active immunization with GST-OspC is practicable to prevent or treat *B. burgdorferi* infection in AKR/N mice. These animals not only develop persistent spirochetal infection, but also clinical arthritis (Table 5, and [24]). In view of the fact that both quality and quantity (Tables 1 and 3; Fig. 1; [16]) of GST-OspC-specific Ab are important for their therapeutic efficacy, we first analyzed the kinetics of specific IgG Ab production generated upon immunization of naive AKR/N mice with GST-OspC (Fig. 2B). Only marginal amounts of OspC-specific IgG Ab ($0.4 \mu\text{g/ml}$) were detectable in one of three mice tested within the first 7 days after priming. Significantly more IgG Ab ($125.9 \pm 64.6 \mu\text{g/ml}$) were seen on day 14 following the first boost (day 7), with peak responses ($410.4 \pm 217.8 \mu\text{g/ml}$) at day 21 after the second boost (day 14), and subsequent decline. However, concentrations of up to $32.2 \pm 16.7 \mu\text{g/ml}$ OspC-specific IgG Ab were detectable for more than 100 days after immunization.

Groups of four to five mice were immunized with GST-OspC in adjuvant s.c. either 30 days before or 1, 10, 22 or 60 days after experimental infection with 10^3 spirochetes.

Table 4. Prevention and resolution of experimental or tick-borne *B. burgdorferi* infection in BALB/c mice by passive transfer of GST-OspC-specific mouse immune serum^{a)}

Infection route	Intervals of IS transfer (days p.i.)	Dose ($\mu\text{g}/\text{mouse}$)	Ear culture (days p.i.)	
			42–58	65–73
Syringe inoculation	None	None	4/5	3/5
	–1 h	10	0/5	ND
	10, 13, 16, 19	30	1/5	ND
	20, 23, 26, 29	30	0/5	ND
	40, 43, 46, 49	30	ND	1/5
Tick-borne infection	None	None	1/5	3/5
	10, 13, 16, 19	30	0/4	0/4
	20, 23, 26, 29	30	0/5	0/5

a) BALB/c mice were treated with OspC-specific mouse IS either before (–1 h) or starting at the indicated time points p.i. (4 \times , 3-days intervals). Recipients were challenged either experimentally with 10^3 cultured spirochetes (ZS7, s.c.) or with previously infected (ZS7) nymphal ticks. Control mice remained untreated.

Control mice were either given rLip-OspA or remained untreated. Serum titers of anti-OspA and anti-OspC Ab were determined in individual mice before and/or after immunization. As shown in Table 5, experimental infection of AKR/N mice alone led to the generation of anti-OspC but not anti-OspA Ab, supporting previous studies [6, 7, 25]. Upon immunization with GST-OspC, all 23 animals produced high amounts of OspC-specific Ab within 20–30 days post immunization, ranging from 38 to 129 $\mu\text{g/ml}$, independent of their state of infection. Similarly, all mice immunized with rLip-OspA generated high amounts of OspA-specific Ab (397–2446 $\mu\text{g Ab/ml}$).

Active immunization of AKR/N mice with either rLip-OspA or GST-OspC prevented subsequent experimental infection (Table 5). In contrast, when AKR/N mice were immunized with either GST-OspC or rLip-OspA starting at either day 1, 10, 22 or 60 p.i., no significant effect on either the onset of clinical arthritis, which was first apparent in infected control mice between days 20–25 p.i., or

on the course of an established clinical arthritis was seen. In addition, with few exceptions none of the four immunization protocols led to clearance of spirochetes from the infected mice. This cannot be due to an inherent inability of OspC-specific Ab to target and kill spirochetes *in vivo* since repeated passive transfer of GST-OspC-specific IS (30 $\mu\text{g Ab}$, 4 \times) from AKR/N mice into C.B-17 SCID mice at day 22 p.i. led to substantial reduction of fully developed clinical arthritis and to elimination of spirochetes within 10 days after the last treatment (data not shown).

In light of the notion that IgG isotypes express distinct potentials to control *B. burgdorferi* infection [5, 8], we compared the Ig isotypes of OspC-specific Ab in pooled IS from infected and subsequently immunized AKR/N mice with those from recipients which were either only immunized with GST-OspC or experimentally infected with 10^3 spirochetes. All IS were shown to contain OspC-specific Ab of the Ig isotypes IgG1, IgG2a, IgG2b and IgG3, though at varying quantities, irrespective of

Table 5. Prevention, but not resolution, of experimental *B. burgdorferi* infection in AKR/N mice by active immunization with GST-OspC^{a)}

Time of immunization (day)	Recombinant vaccine	Anti-OspA IgG ($\mu\text{g/ml}$)		Anti-OspC IgG ($\mu\text{g/ml}$)		Clinical arthritis		Ear culture	
		before immunization	after immunization	before immunization	after immunization	before immunization	after immunization	before immunization	after immunization
-30	None	ND	0	ND	1.0 \pm 0.3	0/4	4/4	ND	4/4
	rLip-OspA	ND	677.6 \pm 41.2	ND	0.2 \pm 0.1	0/5	0/5	ND	0/5
	GST-OspC	ND	0.0 \pm 0.0	ND	37.8 \pm 25.5	0/5	0/5	ND	0/5
1 p.i.	None	ND	0.0 \pm 0.0	ND	10.9 \pm 5.2	0/5	3/5	ND	3/5
	rLip-OspA	ND	396.6 \pm 68.4	ND	10.1 \pm 5.8	0/5	4/5	ND	4/5
	GST-OspC	ND	0.0 \pm 0.0	ND	105.2 \pm 52.6	0/5	4/5	ND	5/5
10 p.i.	None	ND	0.0 \pm 0.0	ND	7.6 \pm 3.6	0/5	4/5	ND	4/5
	rLip-OspA	ND	1463.5 \pm 738.0	ND	5.8 \pm 2.2	0/5	3/5	ND	3/5
	GST-OspC	ND	0.0 \pm 0.0	ND	90.8 \pm 54.0	0/5	4/5	ND	4/5
22 p.i.	None	0.0 \pm 0.0	0.0 \pm 0.0	4.4 \pm 3.6	1.5 \pm 0.3	3/5	4/5	5/5	5/5
	rLip-OspA	0.0 \pm 0.0	1480.0 \pm 248.1	8.7 \pm 3.5	1.3 \pm 0.3	2/3	3/3	2/3	3/3
	GST-OspC	0.0 \pm 0.0	0.0 \pm 0.0	5.4 \pm 1.2	128.8 \pm 22.4	1/4	3/4	2/4	4/4
60 p.i.	None	0.0 \pm 0.0	0.0 \pm 0.0	1.8 \pm 0.4	2.0 \pm 1.1	4/4	4/4	4/4	4/4
	rLip-OspA	0.0 \pm 0.0	2446.3 \pm 606.0	1.1 \pm 0.7	0.7 \pm 0.7	4/4	1/4	4/4	4/4
	GST-OspC	0.0 \pm 0.0	0.0 \pm 0.0	1.7 \pm 1.3	112.0 \pm 37.3	4/4	3/4	4/4	4/4

a) AKR/N mice were immunized with either GST-OspC or rLip-OspA (10 $\mu\text{g/mouse}$, 3 \times , 7 days interval) at the indicated time points. Control mice remained untreated. All mice were experimentally inoculated with 10^3 spirochetes (ZS7, s. c.). Clinical and serological examinations as well as recultivation of spirochetes from ear biopsies were done both at the time points before immunization and/or 20–30 days after the last antigen boost (exception: animals immunized 30 days before infection were examined on day 62 p.i.).

whether mice were only infected or actively immunized before (day 30) or after (day 1, 10, 22 or 60 p.i.) infection (data not shown).

2.4 Kinetics of *ospA* and *OspC* gene expression in infected tissues from infected mice

The efficacy of *OspC*-based therapeutic vaccination critically depends on the accessibility of spirochetes for Ab and the level of *OspC* expression. To investigate the latter aspect, tissue specimens from infected mice were analyzed for the presence of spirochetes and the expres-

sion of *OspC*-specific mRNA using reverse transcription (RT)-PCR. As shown in Fig. 3, *OspA*- and *OspC*-specific mRNA is readily detectable by RT-PCR in low passage cultured spirochetes. The detection limit is 24–120 pg of spirochete-specific cDNA (~30–1200 spirochetes/organ). After experimental infection of C.B-17 SCID and AKR/N mice, *OspC*-specific mRNA could be amplified from distant skin (ear) and heart biopsy specimens from both mouse strains, at the early stage of infection between days 25 and 45 p.i. When analyzed around day 100 p.i., *OspC*-specific mRNA could still be amplified from ear, heart and also from spleen of C.B-17 SCID mice. In contrast, at this time point p.i., *OspC*-

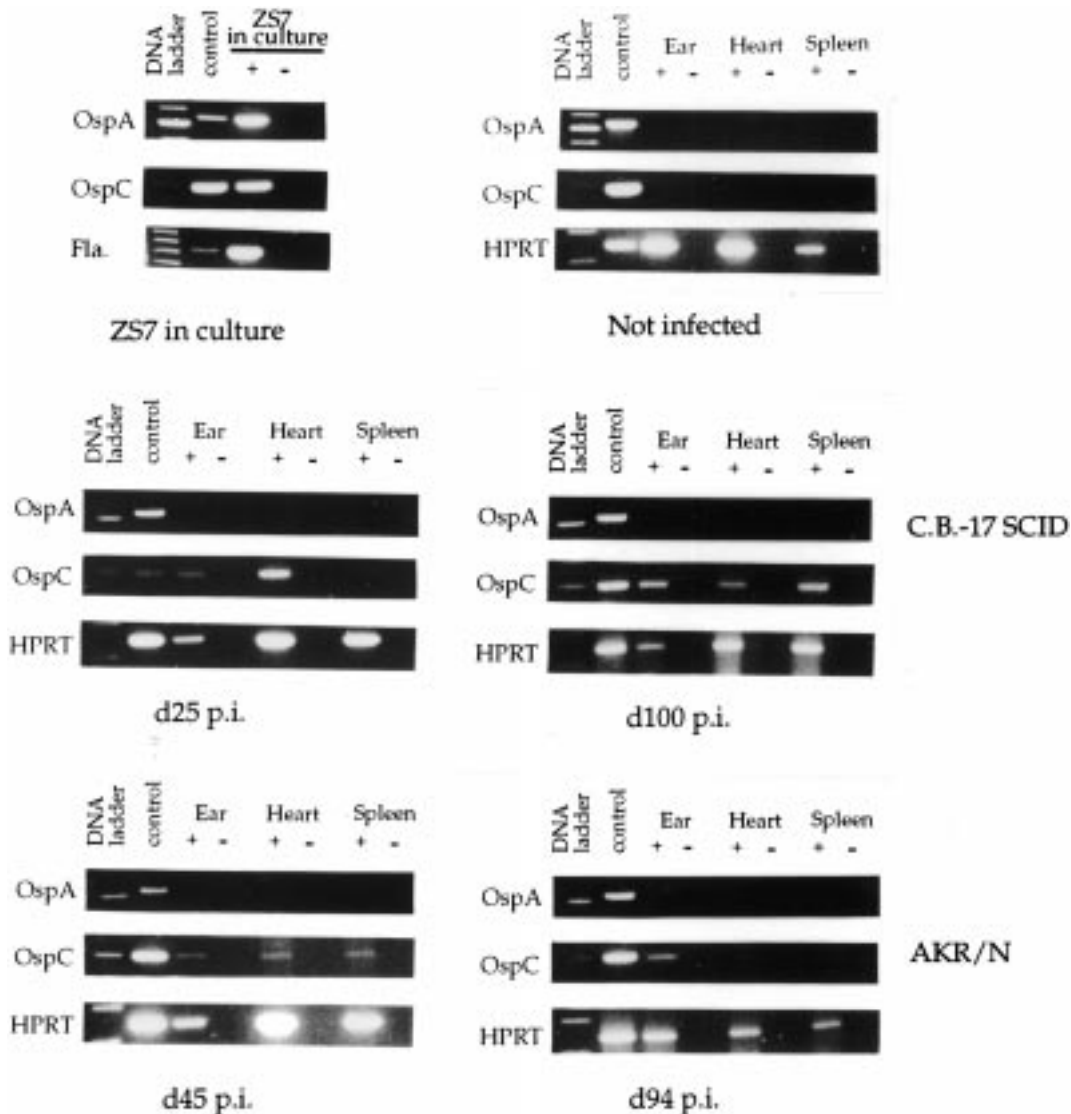


Figure 3. Detection of *ospA* and *OspC* gene expression in tissue specimens from ear, heart and spleen of infected C.B-17 SCID and AKR/N mice at indicated time intervals p.i. by RT-PCR (\pm reverse transcriptase), as described in Sect. 4.9.

specific transcripts were only detected in tissue samples from ear, but not in those from heart or spleen of AKR/N mice. Consistent with serological and molecular genetic data [6, 7, 18, 25, 26], OspA-specific mRNA could not be detected at all in any of the three target organs of both mouse strains between days 45 and 100 p.i. In addition, OspA- and OspC-specific mRNA could not be amplified from the three target organs of uninfected control mice.

3 Discussion

The main findings of the present study are that (1) OspC is consistently expressed by *B. burgdorferi* during infection in mice for at least 100 days p.i., (2) passive transfer of OspC-specific Ab generated against GST-OspC prevents and resolves disease and/or infection in both experimentally and tick-infected immunocompromised (C.B-17. SCID) and immunocompetent (BALB/c) mice, and (3) active vaccination of AKR/N mice with recombinant GST-OspC fusion protein is suitable to prevent, but not to cure, *B. burgdorferi* infection.

From the two structural distinct forms of OspC tested, *i.e.* GST-OspC and its derivative rOspC (lipid free), only the former one elicits protective Ab against infection (Table 1). This is not due to a differential immunogenicity of the two antigen preparations, since both GST-OspC and rOspC induced similar amounts of OspC-specific Ab in mice. It is more likely that both recombinant molecules, which only differ with respect to the GST domain, exhibit distinct conformational epitopes and thus lead to the induction of different arrays of Ab specificities. This assumption is also supported by a recent observation showing that protective OspC-specific Ab were only induced by immunization with a crude *Escherichia coli* lysate containing rOspC, but not with its denatured form, in spite of similar immunogenicity [20]. At present, it is not known to what extent the conformation of GST-OspC corresponds to that of natural OspC and whether the OspC-specific Ab produced during infection are protective at all. However, it is notable that all preparations of recombinant OspC fusion proteins tested so far, including those from European (Pko [11]), ZS7 ([16] and here) and North American isolates (SON 188 [19], B31 [20]), except one strain (N40 [21]), were able to induce protective Ab in mice. This was also true with Ab generated to GST-OspC in rabbits. However, it became clear that the biological activity of the Ab generated to individual recombinant OspC preparations is not only dependent on the immunogenicity of the antigen, but also critically influenced by the nature of OspC as well as the genetic background of the recipient. This is of importance for the design of effective OspC vaccine formulations.

We have previously reported that polyclonal and mono-specific mouse IS to GST-OspC resolve chronic arthritis and carditis and clear spirochetes in experimentally infected C.B-17 SCID mice [16]. The experiments presented now demonstrate that the same protocol is also suitable to eliminate infection both from experimentally and tick-inoculated immunocompetent BALB/c mice. Spirochetes could not be recovered from most of the ear biopsies of infected animals repeatedly treated with OspC-specific IS starting at either days 10, 20 and 40 after syringe inoculation (18/20) or at days 10 or 20 after tick-borne infection (0/9). This is in line with previous reports [16, 18] and indicates that (1) spirochetes express OspC during infection in mice, at least for the time periods tested, independent of whether they were introduced via needle or natural route and (2) that disseminated spirochetes are accessible and susceptible to elimination by OspC-specific Ab. These assumptions are further supported by our findings that OspC-specific mRNA was detectable in biopsy specimens from various organs, including ear, heart and spleen, of immunodeficient (C.B-17 SCID) and immunocompetent (AKR/N) mice for up to 100 days p.i. The fact that none of the *ex vivo* tissue samples contained OspA-specific mRNA corroborates previous studies and emphasized that OspA is not readily expressed during infection in mice [18, 25].

Active immunization of AKR/N mice with GST-OspC only resulted in complete protection against subsequent challenge with *B. burgdorferi*, but failed to clear or modulate established infections. This finding is surprising for two reasons. Firstly, OspC-specific mRNA was observed in tissue specimens from AKR/N ear and heart for up to 45 days p.i. and was still detectable in ear biopsy specimens at day 94 p.i. Secondly, both protocols led to the production of OspC-specific Ab at quantities exceeding those required to resolve chronic *B. burgdorferi* infection in passive transfer experiments. A general unresponsiveness of AKR/N mice to protection-inducing epitopes was excluded by the above results regarding prevention of *B. burgdorferi* infection in AKR/N mice and the finding that OspC-specific IS from GST-OspC immunized but uninfected AKR/N mice were able to prevent and resolve infection upon transfer into C.B-17 SCID mice (data not shown). It is also unlikely that the different protective potential observed with the two OspC-specific Ab populations generated before or after infection is due to distinct patterns of IgG isotypes, and thus distinct biological activities. This is inferred from the data showing that similar patterns of IgG isotypes, including those of IgG1, IgG2a, IgG2b and IgG3, were generated in AKR/N mice in response to GST-OspC, independent of the immunological history (data not shown). Thus, the failure to clear an established infection in subsequently immunized AKR/N mice may be due to a decreased surface expres-

sion of OspC or to sequestration of *B. burgdorferi*, and indicates that spirochetes residing in skin tissue are less vulnerable to Ab-mediated destruction (see also below). This is also interesting since skin seems to be the most prominent tissue for isolation of *B. burgdorferi* sensu lato from reservoir hosts on which ticks feed and from which they get the infection [23, 27, 28].

The failure to resolve an established *B. burgdorferi* infection by active immunization with GST-OspC may also be due to selection of Ab responses to GST-OspC by previous encounter with the native antigen during infection. This phenomenon of priming-induced deflection of antibody responses, termed “original antigenic sin”, was first observed with humans vaccinated with influenza [29, 30]. In these studies the resulting Ab were shown to have higher affinity for strains to which patients had been exposed in childhood than for the vaccine strain. It was subsequently revealed that the immunological response was oriented toward cross-reactive epitopes within the polymorphic viral hemagglutinin with simultaneous ignorance of additional epitopes present in the vaccine [31, 32]. Accordingly, Ab responses to GST-OspC in previously infected mice would also be expected to be mainly directed to cross-reactive epitopes expressed on both native and recombinant OspC protein and not to new ones expressed by the recombinant GST-OspC protein. The finding that protection by OspC-reactive Ab is only achieved upon vaccination of naive, but not infected mice with GST-OspC, suggests that the protective epitope(s) is either not expressed on native OspC or not immunogenic during an ongoing infection. This by itself is of significance for the survival of *B. burgdorferi* in an immunocompetent environment. If this non-responsiveness to protection-inducing epitopes is due to clonal imprinting, injudicious vaccine design will cause problems because of the potential suppression of protective immune responses caused by the pathogen itself. Only the presentation of a synthetic vaccine lacking those OspC epitopes to which the immune system has been imprinted would circumvent this dilemma.

Taken together, the results presented here demonstrate that passive vaccination with OspC Ab is suitable to prevent and cure experimental and tick-borne *B. burgdorferi* infection in mice. This reveals a previously unrecognized role for OspC in protective immunity against *B. burgdorferi* infection and may have important implications for the development of new prophylactic and therapeutic strategies against human Lyme disease.

4 Materials and methods

4.1 Animals

Adult female mice of strains AKR/N (H-2^k), BALB/c (H-2^d) and C.B-17 SCID (H-2^d) were bred under SPF conditions at the Max-Planck-Institut für Immunbiologie, Freiburg, Germany. Animals between 6 and 8 weeks of age were used throughout the experiments.

4.2 Spirochetes and infection of mice with *B. burgdorferi*

The virulent low-passage (two to four *in vitro* passages) tick isolate *B. burgdorferi* ZS7 was grown in Barbour-Stoenner-Kelly medium at 32 °C for 48–72 h and harvested as described [33]. Animals were challenged either by needle inoculation with 1×10^3 viable *B. burgdorferi* organisms (s.c.) or by previously infected *Ixodes ricinus* nymphs, as described [34].

4.3 Recombinant antigens

A full-length rLip-OspA from *B. burgdorferi* strain ZS7 was generated as described [35] and was kindly provided by SmithKline Beecham, Rixensart, Belgium. GST-OspC from *B. burgdorferi* strain ZS7 was generated using established protocols [36]. rOspC was generated by cleaving the GST-OspC fusion protein with thrombin.

4.4 Polyclonal immune sera to recombinant antigens

BALB/c mice were repeatedly inoculated with either 10 µg of rLip-OspA, GST-OspC, rOspC or GST in 100 µl ABM2 adjuvant (Sebak, Aldenbach, Germany) as described [16]. Rabbits (Chinchilla-Bastard) were injected with 400 µg GST-OspC in CFA, directly into popliteal lymph nodes and 200 µg of the same antigen preparation into multiple s.c. sites of the rabbit and boosted once 4 weeks later by injecting 700 µg of the same antigen preparation into multiple s.c. sites. OspA- or OspC-specific Ab were determined with ELISA using either rLip-OspA or rOspC as substrates.

4.5 Analysis of serum Ab by ELISA and Western blot

Serum Ab to *B. burgdorferi*, OspA or OspC were quantified by a solid-phase ELISA as described [37] using as substrates either 10 mg/ml of *B. burgdorferi* lysate or 1 µg/ml of either rLip-OspA (ZS7) or rOspC (ZS7). Western blot analysis, using whole-cell lysate of *B. burgdorferi* strain ZS7 as antigen preparation, was done as described [9].

Isotypes of OspC-specific Ab (IgM, IgG, IgG1, IgG2a, IgG2b and IgG3) were analyzed by Western blot as described [37],

with the modification that rOspC was used as antigen instead of spirochetal lysate. To compare the relative amounts of OspC-specific isotypes in individual serum samples, IS tested were adjusted to contain 200 ng of total OspC-specific Ab for each nitrocellulose strip.

4.6 Passive immunization

C.B-17 SCID and BALB/c immunocompetent mice were either injected i.p. with IS (in some cases with OspA-specific IS) 1 h before infection or were left untreated. Alternatively, mice were first infected s.c. and subsequently given repeatedly (4 × at 3–4 day intervals) various amounts of polyclonal IS specific for either OspA or OspC (i.p.) at indicated time points relative to infection. C.B-17 SCID mice were monitored for the development of clinical arthritis in the tibiotarsal joints under double-blind conditions. The severity of arthritis was scored in the right and left tibiotarsal joint [16]. The status of infection of disease-resistant BALB/c mice was determined by testing for the outgrowth of viable spirochetes from cultured biopsies, as described [22].

4.7 Active immunization

AKR/N mice were immunized s.c. with rLip-OspA or GST-OspC (10 µg/mouse, 3 ×, in 7-day intervals) either before infection or at the indicated time point after experimental infection with culture-derived spirochetes. The course of disease and infection was monitored by determining the severity of clinical arthritis and by testing the outgrowth of viable spirochetes from cultured ear biopsies, as described [16].

4.8 Histology

For histopathological examination, C.B-17 SCID mice were killed at indicated time points p.i. Heart tissues were fixed in 10 % formaldehyde, embedded in paraffin and stained with hematoxylin and eosin. Samples were examined under double-blind conditions. For comparison of the histopathological alterations, in particular, cellular infiltrations, the following scoring system was used: ++, extensive; +, severe; ±, moderate; –, no lesion.

4.9 Detection of *ospA*- and *OspC*-specific mRNA in biopsies by RT-PCR

Untreated and infected C.B-17 SCID and AKR/N mice were killed at indicated time points. Total RNA was isolated from two entire ears, half of the heart and one third of the spleen of individual mice by using RNAClean reagent (AGS GmbH, Heidelberg, Germany). After treatment with DNase (Promega, Madison, WI), 15 µg of ear RNA, 10 µg of heart RNA and 15 µg of spleen RNA were incubated with reverse transcriptase and random hexamer primers (Pharmacia,

Uppsala, Sweden) to complementary DNA (cDNA) using the Superscript II RT kit (Gibco-BRL, Gaithersburg, MD). Because of overlapping bands generated by random primers, heart RNA (5 µg) was transcribed using a 3' OspA primer (sequence see below). As control for DNA contamination, identical first-strand synthesis reactions were performed in the absence of reverse transcriptase. To verify the success of each RT reaction, a cDNA equivalent to 50 ng of mouse tissue RNA was amplified by PCR using oligonucleotide primers for the HPRT gene: 5'-GCT GGT GAA AAG GAC CTC T-3'; and 5'-CAC AGG ACT AGA ACA CCT GC-3'. For amplification of *ospA*- and *OspC*-specific mRNA, cDNA equivalent to 2 mg of total RNA were added, which represented 1/25 ear RNA, 1/70 heart RNA and 1/210 spleen RNA. Sequences of 5' and 3' primers for *ospA* are: 5'-GGG AAT AGG TCT AAT ATT AGC C-3'; 5'-TGC CTG AAT TCC AAG CTG CA-3'; for *OspC* gene: 5'-CTG ATG AGT CTG TTA AAG GGC C-3'; 5'-TTA CCA AGA CTT GCG TGC TC-3'.

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Correspondence: Markus M. Simon, Max-Planck-Institut für Immunbiologie, Stübweg 51, D-79108 Freiburg, Germany.
 Fax: +49-76 15 10 85 29
 e-mail: simon@immunbio.mpg.de

Weimin Zhong's present address: Department of Immunology, St. Jude Children's Research Hospital, 332 North Lauderdale, Memphis, TN 38105, USA