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Weimin Zhong[1], Lise Gern[2], Michael Kramer[3], Reinhard Wallich[3] and Markus M. Simon[1]

[1] Max-Planck-Institut für Immunbiologie, Freiburg, Germany
[2] Institut de Zoologie, Université de Neuchatel, Neuchatel, Switzerland
[3] Institut für Immunologie der Universität Heidelberg, Heidelberg, Germany

T helper cell priming of mice to *Borrelia burgdorferi* OspA leads to induction of protective antibodies following experimental but not tick-borne infection

Antibodies to the outer surface lipoprotein A (OspA) of *Borrelia burgdorferi* confer protection to SCID mice against subsequent tick-borne or experimental infection. However, OspA-specific antibodies are hardly detectable in naturally infected humans, dogs, hamsters and mice. This is most probably due to limited expression of OspA on spirochetes transmitted from the vector to the host. Here we have tested whether T cell priming of mice would lead to the induction of protective OspA-specific antibodies upon infection. It is shown that AKR/N mice, previously immunized with either a single T helper cell peptide of OspA, or a mixture of 27 peptides spanning the entire molecule, develop OspA-specific IgM or IgG antibodies, including those to a prominent protective B cell epitope of OspA, LA-2, within 7 days of infection with low doses (10^3) of culture-derived spirochetes. In marked contrast, the same groups of pre-sensitized mice failed to generate any detectable OspA-specific antibodies after tick-borne infection for more than 40 days after infection. All mice, irrespective of their state of T cell immunity to OspA or the mode of infection, produced similar levels of OspC-specific IgM and IgG antibodies as early as day 14 after infection. None of the mice previously immunized with OspA peptides were protected against experimental infection, in spite of the appearance of protective antibodies. It is clear from these data that, in contrast to culture-derived spirochetes, the naturally transmitted pathogen fails to express OspA within the mammalian host at levels sufficient for induction of B cell responses, even in the presence of pre-activated T helper cells. Together with the fact that OspA-specific antibodies are mainly operative by eliminating spirochetes from the vector during infestation, the data suggest that OspA-vaccination for T helper cell immunity alone is not sufficient to prevent Lyme disease.

1 Introduction

It is now well established that active and passive vaccination using OspA as immunogen leads to full protection of mice against subsequent tick-borne or experimental infection with *Borrelia burgdorferi* [1, 2]. Immunization of mice with either recombinant OspA lipoprotein (rLip-OspA) or plasmid DNA encoding OspA elicits both specific B and T cell responses as well as sustained levels of protective antibodies [2–7], in particular those related to a prominent protective epitope, LA-2 [8, 9]. However, full protection against natural or experimental infection of mice is only achieved when these antibodies are present before or at the time of challenge [1, 10, 11]. This OspA-specific immunity is mainly operative by killing spirochetes within the infecting tick, thereby blocking bacterial transmission from the vector to the host [12, 13].

After natural infection of humans and mice, early IgM and IgG antibody responses to *B. burgdorferi* are mainly directed to flagellin (41 kDa) and various other antigens with molecular masses between 88–95 kDa, 35–39 kDa and 15–25 kDa, including OspC, but not to OspA [14–19]. Whether the absence of humoral immunity to OspA is due to down-regulation of this lipoprotein on spirochetes upon transmission by ticks [13, 20, 21] or to a permanent loss of OspA expression within the vertebrate host, is not clear at present.

We and others have recently found that priming of mice with a synthetic T helper (Th) peptide of OspA leads to enhanced production of protective OspA-reactive antibodies upon boosting with rLip-OspA [4] and to specific seroconversion upon challenge with low doses of cultured *B. burgdorferi* [7]. This led us to investigate whether T cell priming of mice with OspA-related synthetic peptides also results in the induction of protective anti-OspA antibodies after tick-borne challenge and prevention from infection.

2 Materials and methods

2.1 Animals

Adult female AKR/N mice were kept under specific pathogen free conditions at the Max-Planck-Institut für Immun-

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Correspondence: Markus M. Simon, Max-Planck-Institut für Immunbiologie, Stübeweg 51, D-79108 Freiburg, Germany
Fax: +49-761-510-8529
e-mail: simon@immunbio.mpg.de

Abbreviations: aa: Amino acid **rLip-OspA:** Recombinant outer surface lipoprotein A

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biologie, Freiburg, Germany. Animals between 6 and 8 weeks of age were used throughout the experiments.

2.2 Spirochetes and production of recombinant antigens

The virulent low-passage tick isolate *B. burgdorferi* ZS7 was grown in Barbour-Stoenner-Kelly medium at 32 °C for 48–72 h and harvested as described [22]. A full-length recombinant Lip-OspA (rLip-OspA; calculated M_r 34 kDa) from strain ZS7 was kindly provided by Smith Kline Beecham, Rixensart, Belgium. Briefly, the *ospA* gene encoding the lipidated OspA was expressed in *Escherichia coli*. The soluble recombinant products were subsequently purified to homogeneity by gel filtration (HR-4009), butanol extraction and Q-Sepharose chromatography [23].

For expression and purification of OspC-glutathione S-transferase fusion protein, the leader sequence, *i.e.* 19 N-terminal amino acid (aa) residues, of OspC from *B. burgdorferi* ZS7 was deleted. The product was cloned into the BamHI/EcoRI sites of the expression vector pGEX-2T (Pharmacia-LKB, Freiburg, Germany). The rOspC-GST fusion protein was synthesized in *E. coli* DH5α and the antigen was purified as described [24].

2.3 Synthetic peptides

20-mer peptides from OspA of strain ZS7 were prepared by solid-phase synthesis using Fmoc amino acids and DIC/HOBt activation on a multiple peptide synthesizer (SMPS 350, Zinsser Analytic Frankfurt, Germany) as described [25]. Peptide B4 (aa 186–203) has the following aa sequence: TLSKNISKSGEVSVELNDTV. The numbers indicate the aa positions starting from the N terminus of the OspA protein.

2.4 T cell priming *in vivo* and challenge of mice with either culture-derived spirochetes or experimentally infected ticks

AKR/N mice were inoculated s.c. into the base of the tail with 20 μg of peptide B4 emulsified in 100 μl ABM2 adjuvant (Sebak, Aldenbach, Germany) or 54 μg of a mixture of 27 OspA peptides spanning the entire aa sequence of the OspA molecule (2 μg per peptide) in the same volume of ABM2 adjuvant. Control mice received ABM2 alone, or remained untreated. Ten days after T cell priming mice were challenged either s.c. with 1×10^3 viable *B. burgdorferi* (ZS7) or *Ixodes ricinus* nymphs infected with the same spirochete strain as follows: Swiss mice were infected i.p. with 1×10^3 viable *B. burgdorferi* (ZS7). Two months later, uninfected *I. ricinus* larvae (50 per mouse) were attached onto the back of mice (protected within glued plastic bags). During infestation, all mice were kept in individual cages, placed above trays of water to recover ticks. After molting, nymphs were monitored for infection (50 % of the ticks became infected with *B. burgdorferi*), and were then used to infect AKR/N mice (10 nymphs pro mouse) as described above.

2.5 Analysis of immune sera for antibodies against OspA, OspC and against the protective B cell epitope, LA-2

Serum samples were collected at indicated time intervals from the tails of the mice. OspA- and OspC-specific IgG antibodies were measured by a solid-phase ELISA as described [8] with the modification that plates were coated with 1 μg/ml of rLip-OspA and rOspC, respectively.

For determination of OspA-specific IgM antibodies, 96-well microtiter plates (Nunc Immunoplate, Maxisorp, Roskilde, Denmark) were coated with 1 μg/ml of rLip-OspA overnight at 4 °C. After washing with 0.05 % Tween-20 in PBS (PBS-Tween), the plates were blocked with 0.2 % gelatine in PBS for 2 h at room temperature with shaking. Subsequently, the plates were incubated for 2 h with 100 μl of serially twofold diluted immune sera and seven control sera from naive AKR/N mice (beginning at 1:50 in PBS-Tween). After incubation with alkaline phosphatase-conjugated goat anti-mouse IgM (1:1000 in PBS-Tween, Southern Biotechnology Association, Inc., Birmingham, AL) for 90 min, the substrate p-nitrophenyl phosphate (Sigma 104 phosphatase substrate tablets, St. Louis, MO) was added for 15 min in the dark. Finally, the absorbance was read at 490 nm using an ELISA reader. To determine the end-point titer of test sera, the mean and standard deviation (SD) of the seven negative control sera were determined. The final titer of an immune serum is defined as the dilution showing absorbance values ≥ mean plus 2 SD of control sera. Serum LA-2 equivalent antibodies were determined by a mAb LA-2 competition ELISA as described [5].

2.6 Passive immunization and recultivation of spirochetes from biopsies

The 6–8-week-old female C.B.-17 SCID mice (H-2^d) were injected i.p. with either serial dilutions of pooled AKR/N immune sera (100 μl/mouse), mAb LA-2 (5 μg/mouse), or PBS. After 2 h, each recipient was injected s.c. with 1×10^3 *B. burgdorferi* ZS7 into the base of the tail. C.B.-17 SCID mice were monitored for the development of clinical arthritis in the tibiotarsal joints as described [22] and for the presence of spirochetes by cultivation of ear biopsies, taken between days 12 and 96 [5, 26].

3 Results and discussion

It was shown recently that Th cell priming of mice with synthetic peptides of OspA leads to an early and enhanced appearance of OspA-specific antibodies, including those with protective potential [4], when challenged with either rLip-OspA or low doses ($\leq 10^3$) of culture-derived spirochetes [4, 7]. In the present study, we tested whether the same protocol of Th cell priming would also favor induction of OspA-reactive and protective antibodies after tick-borne infection of mice.

Table 1. Amount of OspA-specific IgG antibodies and protective LA-2 equivalents (μg/ml) in sera of AKR/N mice after priming with OspA peptide B4 (aa 186–203) and subsequent challenge with culture-derived *B. burgdorferi* ZS7[a)]

Priming	Day −3 Anti-OspA IgG	Day 20 after infection Anti-OspA IgG	Day 20 after infection LA-2 Eq	Day 45 after infection Anti-OspA IgG	Day 45 after infection LA-2 Eq
ABM2 ($n = 6$)	<0.1	3.1 ± 1.1	0.9 ± 0.4	4.3 ± 3.8	2.3 ± 0.8
B4 + ABM2 ($n = 8$)	<0.1	12.5 ± 7.3	4.4 ± 1.6	17.7 ± 8.9	9.6 ± 5.7
p value[b)]	[c)]	0.0007	0.0007	0.0027	0.007

a) AKR/N mice were primed with 20 μg of OspA peptide B4 s.c. 10 days before challenge. The day of the challenge with spirochetes is defined as day 0. Control sera were taken 7 days after priming with peptide (day −3). After challenge with 1×10^3 *B. burgdorferi* ZS7 s.c. in the base of the tail, the concentration of OspA-specific IgG antibodies as well as the protective LA-2 equivalent IgG antibodies (LA-2 Eq) from individual mouse sera were determined by a quantitative ELISA and a mAb LA-2 competition ELISA at days 20 and 45. The data are expressed as the mean ± SD.

b) p values were obtained by the nonparametric Mann-Whitney test by comparison of B4-primed groups with ABM2 adjuvant-primed control groups. A p value smaller than 0.05 is considered significant.

c) Calculation not possible.

AKR/N mice were immunized with either one of the prominent Th cell-specific peptides of OspA, *i.e.* B4 [4], or a mixture of 27 peptides spanning the entire OspA molecule *in adjuvant* (ABM2), or with ABM2 alone. Untreated mice served as an additional control. Priming of the T cell compartment by peptide preparations was confirmed by showing that enriched $CD4^+$ T cell populations from mice presensitized with peptide B4 (aa 186–203) could be specifically restimulated *in vitro* with the same but not an unrelated peptide, E1 (aa 16–33), of OspA ([4] and data not shown). In an initial experiment, recipients were primed with either B4 (+ ABM2; eight mice) or with ABM2 (six mice) alone and challenged with 10^3 culture-derived spirochetes by needle inoculation. Levels of OspA-specific IgG antibodies were monitored in individual mice at days −3, 20 and 45 after infection. As shown in

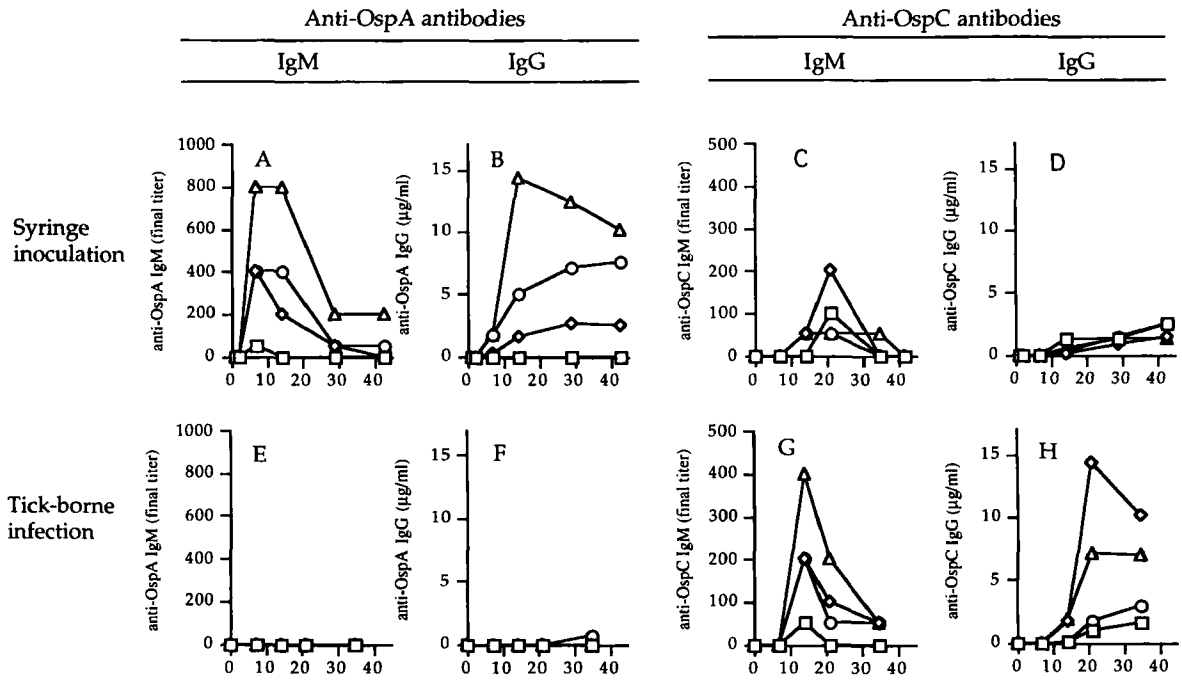

Figure 1. Kinetics of appearance of anti-OspA (A, B, E, F) and anti-OspC (C, D, G, H) antibodies in serum of AKR/N mice previously primed with OspA-related peptide preparations and subsequently challenged with either 10^3 cultured *B. burgdorferi* ZS7 (upper panel) or infected ticks (lower panel). Pretreatment of AKR/N mice: (1) none (squares); (2) ABM2 (100 μl; diamonds); (3) peptide B4 (20 μg in 100 μl of ABM2; circles); or (4) overlapping OspA-related peptide mixture covering the entire OspA molecule (54 μg in 100 μl of ABM2; triangles). Ten days later animals were injected with 1×10^3 *B. burgdorferi* ZS7 s.c. into the base of the tail or naturally infected via *I. ricinus* nymphs infected with *B. burgdorferi* ZS7. IgG antibodies against OspA and OspC were determined from pooled sera (upper panel: 3 mice/group; lower panel: 5 mice/group) by a quantitative ELISA as described in Sect. 2.5. IgM antibodies to OspA and OspC were measured from the same pooled sera by a semi-quantitative ELISA. Results are end-point titers of serum dilutions whose absorbance values are greater than the mean + 2 SD of seven normal sera from naive AKR/N mice.

Table 2. Development of clinical arthritis and persistence of spirochetes in AKR/N mice previously primed with OspA-peptide preparations and subsequently challenged with *B. burgdorferi* ZS7[a)]

	Priming	Clinical arthritis[b)]	Re-cultivation of spirochetes[c)]
Experiment 1	Untreated	3/3	3/3
	ABM2	3/3	3/3
	B4 + ABM2	3/3	3/3
	Peptide mix. + ABM2	3/3	2/3
Experiment 2	ABM2	4/6	6/6
	B4 + ABM2	7/7	8/8

a) Data of experiment 1 and 2 were obtained after syringe inoculation from the mice groups described in Fig. 1 and Table 1, respectively.
b) Development of clinical arthritis was monitored for 58 days (Experiment 1) and 98 days after infection (Experiment 2), respectively (number of mice with clinical tibiotarsal arthritis/number of mice examined).
c) Recultivation of spirochetes from ear biopsies 32 days (Experiment 1) and 45 days after infection (Experiment 2), respectively (number of positive cultures/number of mice tested).

Table 1, at days 20 and 45, the amount of anti-OspA serum IgG antibodies detected was approximately fourfold higher in mice presensitized with B4 + ABM2 as compared to ABM2 alone. No specific antibodies were observed in B4-primed but unchallenged mice (day −3).

In the next step, the kinetics of IgM and IgG antibody responses to OspA were monitored in more detail for up to 42 days after infection in pooled sera from peptide-primed and control AKR/N mice, subsequently challenged with either 10^3 culture-derived spirochetes or with infected *I. ricinus* nymphs. AKR/N mice, previously immunized with either B4 or ABM2 alone, but not untreated control mice, produced similar amounts of OspA-specific IgM antibodies (IgM: dil. 1/400) upon experimental challenge (Fig. 1A). The lack of specificity may be due to an unspecific activation of the IgM response by ABM2. However, upon immunization of mice with a mixture of 27 OspA peptides spanning the entire OspA molecule and including various T cell-reactive epitopes [4], the level of OspA-specific IgM antibodies observed at days 7 and 14 after infection. (IgM: dil. 1/800) significantly exceeded those obtained with B4 + ABM2 or ABM2 alone. From day 14 to day 30 after infection, the amount of OspA-reactive IgM antibodies rapidly declined to baseline levels. When tested for OspA-specific IgG antibodies, significantly higher amounts were found in B4-sensitized as compared to ABM2-treated mice. The respective antibodies were already detectable 7 days after infection. (IgG: 1.7 μg/ml); their level further increased with time, reaching a plateau of ~ 7 μg/ml at day 30 (Fig. 1B). No OspA-specific antibodies were found in B4-primed but unchallenged mice up to 35 days after initial priming (data not shown), demonstrating that peptide B4 alone was unable to induce B cell responses. Immunization of mice with the mixture of OspA peptides resulted in even higher levels of OspA-specific IgG antibodies at day 14 after infection (14.3 μg/ml) which declined with time (Fig. 1B). The remote possibility that the increased amounts of OspA-specific antibodies produced by this protocol is, at least, partially due to previous B cell activation by putative B cell epitopes present in the peptide mixture of OspA could not be excluded. However, the latter protocol did not change the kinetics of appearance of OspA-specific antibodies as observed with B4. In marked contrast, none of the naive or presensitized mice produced detectable amounts of OspA-specific antibodies following tick challenge during the entire observation period (Fig. 1E and F). As expected from previous studies [17, 27], mice of all experimental groups produced significant amounts of OspC-specific IgM and IgG antibodies in response to both, tick-borne (Fig. 1G, H) and culture-derived spirochetes (Fig. 1C, D), as early as 14 days after infection. The level of anti-OspC antibodies was not affected by previous sensitization with OspA peptides. Again, the unspecific activation of the immune system by ABM2 is indicated by the fact that levels of IgM and/or IgG antibodies to OspC were mostly higher in adjuvant-treated as compared to control mice (Fig. 1G, H).

Together, the data allow to draw the following conclusions. First, when spirochetes are inoculated at low doses ($\leq 10^3$), the amount of immunogenic OspA available seems to be limited. An effective induction of anti-OspA antibodies is only achieved in the presence of presensitized T helper cells. This finding confirms and extends previous studies showing that culture-derived spirochetes only transiently express OspA *in vivo* [20] and switch to OspC expression, explaining why experimentally infected mice only seroconverted to OspC, but not to OspA ([17, 18, 21, 27] and data shown here). Obviously, the amount of OspA antigen expressed by the pathogen within mice is too low to induce an antibody response on its own, but is sufficient to do so in the presence of T cell help. Second, tick-transmitted spirochetes are devoid of OspA. Moreover, they do not seem to re-express the antigen in the mammalian host. This assumption is supported by recent studies describing differential expression patterns for OspA on spirochetes in ticks and mice, as determined by mRNA analysis and indirect immunofluorescence: whereas spirochetes express OspA in unfed ticks [20, 28] they rapidly down-regulate this molecule during the course of tick infestation and their concomitant transmission into mice [13, 20]. Furthermore, the biological read-out system of Th cell-supported antibody production used in this study strongly suggests that OspA is absent from spirochetes during the observed period of infection (day 42 after infection) and does not serve as an appropriate target structure for protective antibodies within the mammalien host.

It is well established that *B. burgdorferi* persists in wild rodents and experimental animals in the presence of high concentrations of spirochete-specific antibodies [19, 29–31]. Subsequent studies in mice showed that protective antibodies must be present before or early during infection to readily eradicate spirochetes from hosts [1, 10, 11] and that therapeutic vaccination with OspA does not resolve *B. burgdorferi* infection in mice [32]. Consequently, we monitored the effect of T cell priming on the development of clinical arthritis and clearance of spirochetes upon syringe challenge. The course of clinical arthritis observed in AKR/N mice previously primed with either B4 or the peptide mix was similar to that of control animals, both with respect to the kinetics of the appearance and severity (Table 2, and data not shown). In addition, with one exception (Experiment 1; peptide mix), spirochetes could be recovered from all recipients on day 42 after infection, independent of their pretreatment. Thus, although Th cell priming of mice leads to the induction of OspA-specific antibodies upon experimental challenge [7], the humoral response generated was still inadequate to efficiently control the disease and/or to eliminate spirochetes.

To test whether this was due to the lack of protective antibodies, we determined the amount of LA-2-related antibodies in immune sera of individual B4-primed AKR/N mice upon syringe inoculation. Antibodies to the B cell epitope of OspA, defined by the mAb LA-2, have been shown before to be positively correlated with protection [8, 9, 33]. As seen in Table 1, the amount of LA-2-related antibodies was approximately four times higher in pooled sera from mice pretreated with B4 (+ ABM2) than in mice pretreated with ABM2 alone when tested at days 20 and 45 following experimental challenge. The protective potential of immune sera from B4-primed and ABM2-treated control mice was further evaluated in passive transfer experiments. We found that complete protection of SCID mice against syringe challenge with 10^3 *B. burgdorferi* ZS7 was still achieved with 1/320 diluted immune sera from mice pretreated with B4 + ABM2 but that a dilution of 1/160 was required in case of diluted sera from mice pretreated with ABM2 (data not shown). Thus, Th cell priming of mice to OspA and subsequent experimental challenge leads to induction of OspA-specific antibodies at amounts sufficient to convey protection upon passive transfer into SCID mice. However, for an effective control of an ongoing experimental infection, the critical level of these antibodies seems to accumulate too late.

In conclusion, the results demonstrate that T cell priming to OspA does not lead to induction of OspA-specific antibodies upon tick-borne infection and protection against disease. In addition, the data suggest that vector-derived spirochetes are devoid of OspA and do not re-express this antigen in the mammalian environment, at least during early stages of infection. Together with previous findings that OspA-specific antibodies are only protective when available before or shortly after infection [1, 10, 11] and that they mainly act by eliminating spirochetes within the tick gut [2, 13], the presented data emphasize that OspA-based prevention of Lyme disease is only achieved by vaccination protocols leading to a state of sustained OspA-specific humoral immunity.

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