Oral immunization with recombinant *Yersinia enterocolitica* expressing a measles virus CD4 T cell epitope protects against measles virus-induced encephalitis

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Immunization via the oral route with an attenuated *Yersinia enterocolitica* strain expressing a fragment of the measles virus nucleocapsid protein (aa 79–161) via its type III protein secretion system induced a T helper type 1 response in immunized C3H mice, which conferred protection against measles virus-induced encephalitis in a time- and dose-dependent manner.

Maternal antibodies inhibit vaccine-induced seroconversion after immunization with the attenuated measles virus (MV) vaccine (Katz, 1995). In an experimental model we have shown that a vesicular stomatitis virus expressing the MV haemagglutinin via the respiratory mucosa induces neutralizing antibodies and confers protection after immunization in the presence of MV-specific antibodies (Schlereth et al., 2000). In humans, neutralizing antibodies (Bouche et al., 2002) as well as T cell responses (van Els & Nanan, 2002) seem to be required for protection against MV. In this study, we have tested whether a bacterial vector immunizing via the gastrointestinal mucosa can be used to induce a strong T cell response for possible future application as a co-immunogen for improved immunization in the presence of MV-specific antibodies. As an animal model we used the mouse model of MV-induced encephalitis (MVE) because it has been shown previously that a CD4 T cell response specific for epitope aa 82–98 of the nucleocapsid (N) protein is able to confer protection in the absence of antibodies (Reich et al., 1992; Weidinger et al., 2000). The vector chosen was an attenuated strain of *Yersinia enterocolitica* serotype O8 (isolate WA-314) (Roggenkamp et al., 2001). The attenuated strain *Y. enterocolitica* WA-314 sodA lacks the Mn-cofactored superoxide dismutase and has been shown to colonize the small intestine and Peyer’s patches of C57BL/6 mice but is markedly impaired in its ability to disseminate into internal organs such as liver and spleen (Igwe et al., 1999). Immunization with this mutant results in a strong activation of mucosal immunity and a *Yersinia*-specific T helper type 1 (TH1) response, which protects against challenge with the wild-type *Y. enterocolitica*. Recombinant antigens expressed by *Y. enterocolitica* are presented to CD4 or CD8 T cells, depending on the expression plasmid used (Russmann et al., 2001). This bacterial vector was tested in the mouse model of MVE. C3H mice have been shown to succumb to encephalitis 5–9 days after intracerebral inoculation with the neurotropic, rodent-adapted CAM/RB strain of MV (Niewiesk et al., 1993). Susceptibility is due to a lack of CD4 T cell activation, which results in virus replication and spread in brain tissue. Animals can be protected against MVE either by transfer of MV-specific CD4 T cells or by immunization with the CD4 T cell epitope peptide (aa 82–98) of the MV N protein (Weidinger et al., 2000). Thus, in order to obtain a bacterial vector that was able to induce a protective T cell response after oral immunization, recombinant *Y. enterocolitica* WA-314 sodA strain expressing either the whole MV N or a fragment of MV N containing the CD4 T cell epitope (aa 82–98) were generated.

*Yersinia* expresses a number of virulence-associated proteins, namely the *Yersinia* outer proteins (Yops). Central to the export of Yops is the function of a specialized protein secretion apparatus termed type III. The close contact between *Y. enterocolitica* and the surface of the eukaryotic cell triggers secretion and subsequent translocation of Yops into the cytoplasm of host cells. YopE is a 25 kDa protein containing an N-terminal secretion domain of 15 amino acids and a translocation domain of 50 amino acids. Plasmid pHR43 (Russmann et al., 2001) carries the genetic information for YopE with a deletion of aa 19–73 (YopE<sub>19-73</sub>). This truncated version of YopE lacks part of its translocation domain and is therefore secreted but not translocated into the cytoplasm of eukaryotic cells. The full-length gene of MV N was amplified and inserted into pHr43. The N protein was expressed in large amounts in bacterial cells but no secretion could be detected (data not shown). This was probably due to the fact that N proteins form a large nucleocapsid in the bacterial cell, which subsequently cannot be secreted. As secretion of protein is crucial to...
induce a T cell response, a gene fragment encoding aa 79–161 from MV N was instead amplified from plasmid pSC-N (forward primer BamHI 5'-ATAGGATCC-TTGGATCCCTGTCCTCC-3', reverse primer BamHI 5'-GT-TGGATCCCTGTCCTCC-3') (Tober et al., 1998) and inserted into the BamHI site of pHR43 to create pHR356 (Fig. 1a).

The detection of expression, secretion and translocation of the hybrid YopE–MV-N (aa 79–161) protein was carried out by Western blotting, as described previously (Russmann et al., 2001). To measure expression of YopE–MV-N (aa 79–161), a lysate from non-cell-associated bacteria was used. To measure secretion and translocation, macrophage-like cells were infected with 10% TCA (Triton X-100-soluble fraction). Samples were separated by 13% discontinuous SDS-PAGE and transferred to nitrocellulose membranes as described previously (Russmann et al., 2001). Hybrid YopE–MV-N (aa 79–161) protein was detected by immunoblot analysis by treating the membrane with a polyclonal antibody against YopE (Jacobi et al., 1998) followed by horseradish peroxidase-labelled anti-rabbit antibody. Blots were developed using a chemiluminescence kit. Western blot analysis demonstrated that YopE–MV-N (aa 79–161) was expressed in large quantities in Y. enterocolitica WA-314 sodA (pHR356) (Fig. 1b, lane 1) and was secreted into the supernatant of eukaryotic cells to which bacteria were attached (Fig. 1b, lane 2), but was not translocated into the cytoplasm (Fig. 1b, lane 3). In contrast, wild-type YopE (without a deletion in the translocation domain) was translocated into the cytoplasm (Fig. 1b, lane 3). Therefore Y. enterocolitica WA-314 sodA (pHR356) appeared to be a suitable vector to secrete large amounts of YopE–MV-N (aa 79–161) and stimulate a MV-specific T cell response.

The Y. enterocolitica WA-314 sodA mutant strain is tolerated by different inbred mouse strains to various degrees after oral inoculation with 10^7 c.f.u. (Igwe et al., 1999); BALB/c mice with 10^4–10^5 c.f.u.; H. Russmann, unpublished data). To investigate the susceptibility of C3H mice, animals were fed 3 x 10^3, 3 x 10^5 or 3 x 10^7 c.f.u. of Y. enterocolitica WA-314 sodA (pHR356) in a 30 μl volume of PBS using a microlitre pipette. After inoculation, no clinical signs were observed.

Ten days after oral immunization with 3 x 10^7 c.f.u. Y. enterocolitica WA-314 sodA, 3 x 10^3, 3 x 10^5 or 3 x 10^7 c.f.u. Y. enterocolitica WA-314 sodA (pHR356), mice were challenged intracerebrally with 10^6 TCID50 CAM/RB MV and 4 days later the Yersinia- and MV-specific proliferative T cell response and cytokine secretion were tested. Spleen cells (5 x 10^5) were dispensed in a 200 μl volume of RPMI containing 2% mouse serum with or without 10 μg gradient-purified UV-inactivated MV antigen ml^-1 or 10 μg heat-inactivated Yersinia lysate ml^-1. After 48 h, 0.5 μCi [3H]thymidine was added and cells were harvested 16 h later on to filters. Thymidine incorporation was measured using a betaplate scintillation counter (Weidinger et al., 2000). Immunization with either 3 x 10^7 c.f.u. Y. enterocolitica WA-314 sodA or 3 x 10^3 or 3 x 10^5 c.f.u. Y. enterocolitica WA-314 sodA (pHR356) resulted in a strong Yersinia-specific T cell proliferation (stimulation index of 30–40), in agreement with published results (Igwe et al., 1999). At an inoculation dose of 3 x 10^7 c.f.u. Y. enterocolitica WA-314 sodA (pHR356), Yersinia-specific T cell proliferation was not above background levels seen with spleen cells from naive animals (Fig. 2).

Immunization with either 3 x 10^7 c.f.u. Y. enterocolitica WA-314 sodA (pHR356) or 3 x 10^5 c.f.u. Y. enterocolitica...
WA-314 sodA did not induce a MV-specific T cell proliferation. In contrast, immunization with $3 \times 10^5$ and $3 \times 10^7$ c.f.u. Y. enterocolitica WA-314 sodA (pHR356) induced a low level of MV-specific T cell proliferation, which was statistically significantly higher than that following immunization with $3 \times 10^5$ c.f.u. Y. enterocolitica WA-314 sodA ($P \leq 0.05$ and $P \leq 0.02$, respectively; two-tailed t-test). This level of T cell response was comparable with that seen after intraperitoneal inoculation of $10^6$ p.f.u. MV and has previously been shown to be at least partially protective against MVE (Weidinger et al., 2000; Weidinger, 2002).

After immunization with $3 \times 10^7$ c.f.u. Y. enterocolitica WA-314 sodA (pHR356), supernatants from spleen cells stimulated with Yersinia or MV antigen were tested by ELISA for interferon-$\gamma$ and IL4 secretion (Weidinger et al., 2001). Monoclonal antibody pairs for a sandwich ELISA for the detection of IFN-$\gamma$ (clone R4-6A2 and XMG1.2) and IL4 (clone 11B11 and BVD6-2G2) as well as the respective recombinant cytokines as standards were obtained from Pharmingen. Cytokine ELISA was performed according to the manufacturer’s recommendations. The strong Yersinia-specific T cell proliferation (Fig. 2) correlated with the secretion of IFN-$\gamma$ (22 μg per $10^6$ spleen cells) whereas no IL4 was found (data not shown). Stimulation with MV led to secretion of IFN-$\gamma$ (6 μg per $10^6$ spleen cells) but not of IL4, clearly indicating a TH1 response. It is of interest to note that high-dose inoculation with $10^7$ p.f.u. MV induces a TH2 response in C3H mice (Finke et al., 1995; Weidinger et al., 2001), whereas high-dose inoculation of $3 \times 10^7$ c.f.u. of the recombinant Y. enterocolitica WA-314 sodA (pHR356) strain resulted in a TH1 response. Obviously, induction of the Yersinia-specific TH1 response also directed the MV-specific TH response. Because the MV N fragment secreted by Y. enterocolitica WA-314 sodA (pHR356) contains the aa 81–88 epitope for K2-restricted cytotoxic T lymphocytes (CTLs), the induction of CTLs was also tested. Spleen cells from immunized animals were stimulated in vitro with peptide aa 81–88 and 5 days later tested for cytotoxicity in a chromium-release assay using L929 cells infected with a vaccinia virus recombinant expressing the MV N protein and peptide-pulsed cells as described (Neumeister & Niewiesk, 1998). However, no cytotoxicity was observed (data not shown), indicating that the secreted N fragment induced a CD4 T cell response only. This is in keeping with the fact that YopE–MV-N (aa 79–161) is only secreted but not translocated into the cytoplasm of eukaryotic cells.

In order to test whether the T cell response induced by oral inoculation of mutant Y. enterocolitica WA-314 sodA (pHR356) was protective against MVE, C3H mice were orally immunized with Yersinia and challenged by intracerebral inoculation with $10^5$ TCID50 of the neurotropic rodent-adapted MV CAM/RB strain. Mice were weighed and their reflexes tested every day, as previously described (Weidinger et al., 2000), and moribund animals were killed. Seven days after immunization with either $2 \times 10^5$ c.f.u. Y. enterocolitica WA-314 sodA (pHR356) or Y. enterocolitica WA-314 sodA, animals were not protected against infection with CAM/RB (data not shown). In contrast, 10 days after immunization with $2 \times 10^7$ c.f.u. Y. enterocolitica WA-314 sodA (pHR356), animals were found to be protected against intracerebral challenge (Fig. 3). The level of protection against encephalitis was comparable with peptide immunization or adoptive transfer of MV-specific CD4 T cells (Weidinger et al., 2000, 2001). An immunization dose of

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**Fig. 2.** Induction of Yersinia- and MV-specific T cell responses by Y. enterocolitica WA-314 sodA (pHR356). C3H mice were orally immunized with $3 \times 10^5$, $3 \times 10^6$ or $3 \times 10^7$ c.f.u. Y. enterocolitica WA-314 sodA (pHR356), $3 \times 10^7$ c.f.u. Y. enterocolitica WA-314 sodA or immunized intraperitoneally with $10^6$ p.f.u. MV (Edmonston strain). After 10 days, infected animals and naive control animals were infected intracerebrally with $10^4$ TCID50 CAM/RB MV and 4 days later the proliferative T cell response of spleen cells against $10 \mu g$ heat-inactivated Yersinia lysate ml⁻¹ and 10 μg gradient-purified UV-inactivated MV antigen ml⁻¹ was measured. The extent of MV-specific T cell response was tenfold lower than that for the Yersinia-specific response. Immunization with $3 \times 10^5$ or $3 \times 10^7$ c.f.u. of Y. enterocolitica WA-314 sodA (pHR356) was compared with immunization with $3 \times 10^5$ c.f.u. Y. enterocolitica WA-314 sodA by a two-tailed t-test. SI, Stimulation index.
Fig. 3. Protection of C3H mice against MV-induced encephalitis by Y. enterocolitica WA-314 sodA (pHR356). Ten days after oral inoculation of C3H mice with different doses of Y. enterocolitica WA-314 sodA (pHR356) and Y. enterocolitica WA-314 sodA, animals were infected intracerebrally with $10^6$ TCID$_{50}$ CAM/RB MV. Animals were weighed daily and checked for clinical signs, and moribund animals were sacrificed. Each group contained ten C3H mice. Data for the animals immunized with Y. enterocolitica WA-314 sodA were pooled from two groups (ten animals each) immunized with either $2 \times 10^5$ or $2 \times 10^7$ c.f.u.

2 $\times 10^5$ c.f.u. Y. enterocolitica WA-314 sodA (pHR356) resulted in partial protection, whereas immunization with $2 \times 10^7$ c.f.u., which did not induce a MV CD4 T cell response (see Fig. 2), did not protect against challenge (Fig. 3).

These data indicate that oral immunization with Yersinia leads to a more efficient protective immunity compared with immunization with other bacterial vectors. Using the MVE model in the mouse, it has previously been shown that a recombinant Bacille Calmette-Guerin (Mycobacterium tuberculosis) expressing the full-length MV N protein inside the bacterial cell induced only partial protection (44%) (Fennelly et al., 1995). To confer this partial protection, mice were immunized twice intraperitoneally with a 3-week interval and challenged 6 weeks after the first immunization. In a previous study, we have attempted to use oral immunization with Salmonella AroA mutants secreting MV N T cell epitope aa 79–99 and MV fusion protein B cell epitope aa 404–414 (Spreng et al., 2000). Oral immunization with both constructs at $2.5 \times 10^6$ c.f.u. each was given three times with a 2-week interval. However, challenge 6 weeks after the first immunization resulted only in partial protection (30%). In comparison, animals immunized with Y. enterocolitica WA-314 sodA (pHR356) generated a protective immune response between day 7 and 12 after a single immunization. A possible explanation for the superior induction of protection by Yersinia is the fact that YopE–MV-N (aa 79–161) is secreted very efficiently in large quantities via its type III secretion system and that the recombinant fusion protein between YopE and the N fragment stabilizes the antigen so that it persists longer in the organism to induce a protective T cell response.

One concern raised in respect of subunit vaccines against MV is the occurrence of atypical measles where immunization with a killed vaccine has led to severe measles after contact with wild-type virus (Hilleman, 2001). Although the reasons for this condition are not understood, there is some evidence that the induction of a TH2 response and immune complex depositions are crucial in this context (Polack et al., 1999, 2002). As the vector system used in this study induces a strong TH1 response, it might be beneficial in directing T cell responses towards a protective rather than atypical response.

In summary, our data demonstrate that a single oral immunization via the mucosa with attenuated Y. enterocolitica is able to induce a T cell response protective in a time- and dose-dependent manner against viral infection of the central nervous system. Because Y. enterocolitica induces a mucosal and systemic immune response and due to its good protective capacity in the mouse model, it would be worth while testing this vector system in the presence of maternal antibody.

REFERENCES


