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., 2000). 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 pH43 (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 pH43. 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 cloned into the translocation domain. The nucleotide sequence aa 19–73 in the translocation domain was inserted into the BamHI site to form pH356. (b) Plasmid pH356 was transformed into Y. enterocolitica WA-314 sodA. Expression of wild-type YopE (25 kDa) and recombinant YopE–MV-N (aa 79–161) (27 kDa) was tested from bacterial lysates (lane 1). Secretion of these proteins was tested from supernatants of infected cells (lane 2) and translocation into the cytoplasm from cytoplasmic fractions of P388D1 cells (lane 3).

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 P388D1 cell monolayers were infected with Y. enterocolitica WA-314 sodA (pHR356) at an m.o.i. of 10 for 60 min at 37°C. This resulted in extracellular adhesion of the bacteria to the surface of the macrophages. After infection for 1 h, supernatant was collected and secreted YopE–MV-N (aa 79–161) was precipitated by addition of 10% trichloroacetic acid (TCA). Infected P388D1 cells were incubated for 30 min with Click RPMI 1640 medium containing 100 µg gentamicin ml⁻¹ to kill extracellular bacteria. Cells were then treated with 30 µg proteinase K ml⁻¹ for 15 min at 28°C to eliminate cell-surface-associated Yops. Cells were lysed and proteins were precipitated in the presence of 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.

Ten days after oral immunization with 3×10⁷ c.f.u. Y. enterocolitica WA-314 sodA, or 3×10³, 3×10⁵ or 3×10⁷ c.f.u. Y. enterocolitica WA-314 sodA (pHR356), mice were challenged intracerebrally with 10⁶ TCID₅₀ CAM/MB MV and 4 days later the Yersinia- and MV-specific proliferative T cell response and cytokine secretion were tested. Spleen cells (5×10⁶) 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 3×10⁷ c.f.u. Y. enterocolitica WA-314 sodA or 3×10⁸ or 3×10⁶ 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 3×10⁷ 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 3×10⁷ c.f.u. Y. enterocolitica WA-314 sodA (pHR356) or 3×10⁵ c.f.u. Y. enterocolitica

![Fig. 1. Generation of a recombinant Y. enterocolitica WA-314 sodA (pHR356) strain expressing fragment aa 79–161 of the measles virus nucleocapsid protein (MV N). (a) Plasmid pH43 carries the gene sequence for the YopE protein with a deletion of aa 19–73 in the translocation domain. The nucleotide sequence aa 79–161 from MV N was cloned into the BamHI site to form pH356. (b) Plasmid pH356 was transformed into Y. enterocolitica WA-314 sodA. Expression of wild-type YopE (25 kDa) and recombinant YopE–MV-N (aa 79–161) (27 kDa) was tested from bacterial lysates (lane 1). Secretion of these proteins was tested from supernatants of infected cells (lane 2) and translocation into the cytoplasm from cytoplasmic fractions of P388D1 cells (lane 3).](image-url)
WA-314 sodA did not induce a MV-specific T cell proliferation. In contrast, immunization with 3 × 10^5 and 3 × 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 × 10^5 c.f.u. Y. enterocolitica WA-314 sodA (P ≤ 0.05 and P ≤ 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 × 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-γ and IL4 secretion (Weidinger et al., 2001). Monoclonal antibody pairs for a sandwich ELISA for the detection of IFN-γ (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-γ (22 μg per 10^6 spleen cells) whereas no IL4 was found (data not shown). Stimulation with MV led to secretion of IFN-γ (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 10^6 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 × 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 K^b)-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 TCID_50 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 × 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 × 10^6 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
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 with the mucosa with attenuated *Yersinia 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 *Yersinia 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


