The onset of type 1 diabetes in NOD mice is delayed by oral administration of a bacterial extract (OM-85) and can be completely prevented by its intraperitoneal administration. Optimal prevention is observed when starting treatment at 3 or 6 weeks of age, and some effect is still observed with treatment at 10 weeks of age. Using genetically deficient mice and cytokine-neutralizing monoclonal antibodies, we demonstrate here that the therapeutic effect does not involve T-helper type 2 cytokines (interleukin [IL]-4 and -10) but is tightly dependent on transforming growth factor (TGF)-β. Natural killer T-cells also participate in the therapeutic effect because CD1d−/− NOD mice are partially resistant to the protective effect of OM-85. The question remains of the specificity of the protective effect of OM-85, which may include proinflammatory components. It will thus be important to further characterize the molecular components that afford protection from type 1 diabetes. Lipopolysaccharide is excluded, but other Toll-like receptor (TLR) agonists could be involved because OM-85 stimulated dendritic cells and induced TGF-β production by splenocytes in a TLR-2−, TLR-4−, and MyD88-dependent fashion. Diabetes 55:179–185, 2006

The incidence of type 1 diabetes is steadily increasing in Western countries (1). There is a clear parallel trend toward decreased infectious load in these countries linked to improved hygiene and medical care, including extensive usage of vaccinations and antibiotics (2–3). A similar association is observed in animal models of the disease, such as nonobese diabetic (NOD) mice and Bio-Breeding (BB) rats. The causal relationship between the low rate of infections and high disease incidence was directly demonstrated in the NOD mouse by the increased disease incidence observed after decontamination of mice bred in conventional facilities (2) and by the disease prevention achieved by deliberate infection with a variety of bacteria, viruses, and parasites (4–9). The mechanisms mediating the protective effect of infections on type 1 diabetes are still ill defined.

Here, we present a systematic analysis of the potential mechanisms of diabetes protection in NOD mice afforded by OM-85, an extract manufactured from bacterial species frequently responsible for respiratory tract infections. We took advantage in this study of the availability in the laboratory of several strains of genetically deficient IL4−/− and CD1d−/− NOD mice and of antibodies that neutralize transforming growth factor (TGF)-β and block the interleukin (IL)-10 receptor. The results obtained indicate the multifactorial nature of diabetes protection by OM-85, involving TGF-β and natural killer T (NKT) cells.
OM-85 oral administration. Each mouse received 1, 10, or 25 mg/day of OM-85 active principle orally, 5 days a week for 10 or 30 weeks. The bacterial extract was dissolved in drinking water. Control mice received drinking water without OM-85.

OM-85 intraperitoneal administration. For intraperitoneal injection, fresh solutions of OM-85 were dissolved in saline (NaCl 0.9%). Each mouse received 1 mg of OM-85 active principle per injection, 5 days a week for 10 weeks.

Different compounds were used as controls. In some experiments control mice received intraperitoneal injections of saline without OM-85. In addition, two proinflammatory compounds devoid of any bacterial component, namely, incomplete Freund’s adjuvant (IFA) and N-acetylmuramyl-t-alanyl-d-isoglutamyl-meso-tetradecylglycine (most commonly termed muramyl dipeptide [MDP]) (10–12), served as control treatments. In these experiments each NOD mouse received at 4 or 10 weeks of age a footpad injection of either MDP (25 μg dissolved in NaCl) emulsified in 50 μl IFA or saline emulsified in 50 μl IFA. As a positive control for IFA and MDP + IFA, some NOD mice also received a footpad injection of a complete Freund’s adjuvant (CFA) emulsion according to an identical experimental protocol (at 4 or 10 weeks of age), as previously described (13).

Anticytokine treatment. When needed mice were treated with the 2G.7 monoclonal antibody to human TGF-β that cross-reacts and neutralizes in vivo murine TGF-β (14) (provided by C.J.M. Melief, Leiden University Medical Center, Leiden, the Netherlands) or with a rat monoclonal antibody specific to the mouse IL-10 receptor (15) (provided by A. O’Garra, DNAX, Palo Alto, CA). The antibodies were administered at a dose of 1 mg ⋅ mouse⁻¹ ⋅ injection⁻¹ i.p. three times a week for 4 weeks.

Limulus amebocyte assay. The absence of any significant endotoxin activity in OM-85 preparation was checked by a limulus amebocyte assay (Invitrogen, Clergy Pontoise, France) following the manufacturer’s recommendations.

Preparation and culture of dendritic cells. Primary bone marrow dendritic cells were isolated from femora of wild-type or genetically Toll-like receptor (TLR)- or MyD88-deficient mice. Briefly, bone marrow was flushed out with Hank’s balanced salt solution supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. Cells were cultured at 2 × 10⁵ cells/ml and per well in RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 mmol/l L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, 0.5 mmol/l β-mercaptoethanol, 0.1 mg/ml gentamicin, and 10% supernatant of XR663 cell line–secreting granulocyte macrophage colony stimulating factor. Fresh medium was added every 3 days. At day 9, immature dendritic cells were collected and stimulated by different concentrations of OM-85 in fresh medium for 24 h. Supernatants were then collected and assayed using a murine IL-12p40 enzyme-linked immunosorbent assay kit (R&D Systems, Lille, France).

Cell recovery and T-cell assays. For analysis of cytokine production, total splenocytes or purified T-cell subsets were cultured in triplicate in 96-well flat-bottom microtiter plates (Costar, Cambridge, MA) at 2 × 10⁶ cells/well in the presence of OM-85 (at different doses as indicated in figure legends) or medium alone. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO₂ in RPMI supplemented with 1% nonessential amino acid, 1% minimum essential medium sodium pyruvate, 0.5 mmol/l β-mercaptoethanol, 0.1 mg/ml gentamicin, and 10% supernatant of XR663 cell line–secreting granulocyte macrophage colony stimulating factor. Fresh medium was added every 3 days. At day 9, immature dendritic cells were collected and stimulated by different concentrations of OM-85 in fresh medium for 24 h. Supernatants were then collected and assayed using a murine IL-12p40 enzyme-linked immunosorbent assay kit (R&D Systems, Lille, France).

Enzyme-linked immunosorbent assay for cytokine production. For cytokine measurement Duoset kits quantifying TGF-β, IFN-γ, IL-4, IL-12p40, and IL-10 (R&D Systems) were used. Recombinant cytokines were used as standards. In vitro stimulation assays were repeated at least three times. Measurements were performed in duplicate or triplicate. Data are the means ± SE.

Statistical analysis. Diabetes incidence was plotted using the Kaplan-Meier method, i.e., nonparametric cumulative survival plot. Statistical comparisons between the curves were performed using the log rank (Mantel-Cox) test, which provided the corresponding χ² values. When needed, statistical comparison of mean values was performed using Student’s t test.

RESULTS

Diabetes prevention after intraperitoneal administration of OM-85. In our colony, female NOD mice spontaneously develop overt diabetes starting at 14–16 weeks of age (usually with an incidence of ~80% at 35 weeks of age). Female NOD mice were treated with OM-85 starting at 3, 6, 10, or 15 weeks of age. The product was administered at a dose of 1 mg/day i.p. of active principle, 5 days a week for 10 weeks.

In the group of mice injected with OM-85 from 3 weeks of age, a significant and long-term protection from diabetes was observed (Fig. 1A and Table 1). A similar protection was observed when treatment was started at 6 weeks of age (Fig. 1B) (experimental groups treated with OM-85 included six mice each; the control group included five mice). When treatment was started at 10 or 15 weeks of age, a partial though nonsignificant protection was observed.
As additional controls, in parallel to saline, we tested two proinflammatory compounds, namely, IFA and synthetic MDP. MDP is a component of the mycobacterial cell wall, initially characterized by Lederer and Chedid and colleagues (10–11), which is nonimmunogenic per se while expressing proinflammatory adjuvant-like properties. In fact, it acts as an hapten when conjugated with various carriers (10–12,16–18). As a positive control for IFA and MDP+IFA, NOD mice were also treated with a CFA emulsion as previously described (13). Importantly, no protection was observed after parenteral administration of IFA or MDP+IFA in experimental conditions where CFA showed full protective activity (10 mice were included in each experimental group) (Fig. 2). One may also note that CFA, like OM-85, is active when treatment is started at a young age, but it loses most of its activity when given at later disease stages.

**Effect of oral administration.** Mice were treated orally with different doses of OM-85 (1, 10, or 25 mg/day of active principle), 5 days a week for 10 or 30 weeks. The treatment was started at 3, 6, 10, or 15 weeks of age. Results of several experiments showed some variability. Consistent, though statistically borderline, protection (P = 0.056) was obtained after administration of 25 mg OM-85 for 10 weeks, starting treatment at 6 weeks of age (each experimental group—OM-85 and controls—included seven mice) (Fig. 1C). Some protection was also observed with the 10-mg dose, starting treatment at 6 weeks of age. No effect was observed with the 1-mg dose, even when starting treatment at 3 weeks of age (P = 0.477), a schedule fully efficacious when using the intraperitoneal route (Fig. 1A).

**Diabetes protection by OM-85 does not depend on T-helper type 2 cytokines.** IL-4 dependency was studied by comparing the effect of 1 mg OM-85 administered intraperitoneally in IL-4−/− and wild-type NOD mice. A similar protection was observed in the two strains (OM-85–treated NOD IL-4−/−: n = 9; control NOD IL-4−/−: n = 6; no significant difference, P = 0.216) (Fig. 3A), thus suggesting that IL-4 is not implicated in the therapeutic effect of OM-85.

To study the role of IL-10 on the effect of OM-85 in type 1 diabetes, OM-85 was administered intraperitoneally in NOD mice from 3 to 13 weeks of age, followed by administration of a monoclonal antibody directed to the IL-10 receptor. The antibody was given at a dose of 1 mg three times a week, from 13 to 16 weeks of age. As shown in Fig. 3B, the treatment did not abrogate the protective effect of OM-85 (OM-85–treated NOD: n = 10; OM-85 + anti–IL-10 receptor–treated NOD: n = 7; control NOD: n = 11; P = 0.600). Similar data were obtained in a second experiment (data not shown).

**Contribution of TGF-β to the protective effect of OM-85.** To analyze the possible role of TGF-β in the OM-85–mediated protection of diabetes, treatment with OM-85 (1 mg/day i.p. from 3 to 13 weeks of age) was followed by the administration of a neutralizing anti–TGF-β antibody, given at the dose of 1 mg i.p. three times a week, from 13 to 16 weeks of age. Results shown in Fig. 3C clearly indicate that anti–TGF-β abrogated the protective effect of OM-85 (OM-85–treated NOD: n = 10; OM-85 + anti–TGF-β–treated NOD: n = 6; control NOD: n = 11; P = 0.026), thus suggesting that OM-85–mediated protection is a TGF-β–dependent active phenomenon. Similar data were obtained in a second experiment (data not shown).

**Role of NKT cells in OM-85–mediated protection.** CD1d−/− mice are devoid of NKT cells, whose intrathymic selection depends on CD1d (19). CD1d−/− NOD mice were treated with OM-85 (1 mg/day i.p., 5 days a week for 10 weeks, starting treatment at 3 or 4 weeks of age, depending on the experiment). In both experiments shown in Fig.
that CD1d\(^{-/-}\) and wild-type NOD mice were treated with OM-85 (1 mg i.p./day). A similar diabetes incidence was observed between the two groups (OM-85-treated NOD IL-4\(^{-/-}\): n = 9; and control NOD IL-4\(^{-/-}\): n = 6). B: Treatment with anti–IL-10r antibody (1 mg i.p. three times a week) did not reverse protection induced by OM-85 (1 mg/day i.p.). (OM-85–treated NOD: n = 10; OM-85 + anti–IL-10r–treated NOD: n = 7; and control NOD: n = 11). C: Treatment with a neutralizing anti–TGF-\(\beta\) antibody (1 mg i.p. three times a week) effectively reversed protection induced by OM-85 (1 mg/day i.p.). (OM-85–treated NOD: n = 10; OM-85 + anti–TGF-\(\beta\)–treated NOD: n = 8; and control NOD: n = 11).

FIG. 3. Major role of TGF-\(\beta\) in the protective effect of OM-85. A: Female IL-4\(^{-/-}\) and wild-type NOD mice were treated with OM-85 (1 mg i.p./day). A similar diabetes incidence was observed between the two groups (OM-85–treated NOD IL-4\(^{-/-}\): n = 9; and control NOD IL-4\(^{-/-}\): n = 6). B: Treatment with anti–IL-10r antibody (1 mg i.p. three times a week) did not reverse protection induced by OM-85 (1 mg/day i.p.) (OM-85–treated NOD: n = 10; OM-85 + anti–IL-10r–treated NOD: n = 7; and control NOD: n = 11). C: Treatment with a neutralizing anti–TGF-\(\beta\) antibody (1 mg i.p. three times a week) effectively reversed protection induced by OM-85 (1 mg/day i.p.). (OM-85–treated NOD: n = 10; OM-85 + anti–TGF-\(\beta\)–treated NOD: n = 8; and control NOD: n = 11).

FIG. 4. Involvement of NKT cells in the diabetes protection afforded by OM-85 treatment. Female CD1d\(^{-/-}\) or wild-type NOD mice were treated intraperitoneally with OM-85 (1 mg/day, 5 days a week). Results from two independent experiments are detailed in A (OM-85–treated NOD CD1d\(^{-/-}\): n = 10; control NOD CD1d\(^{-/-}\): n = 10; OM-85–treated NOD: n = 10; and control NOD: n = 11) and B (OM-85–treated NOD CD1d\(^{-/-}\): n = 5; control NOD CD1d\(^{-/-}\): n = 11; OM-85–treated NOD: n = 5; and control NOD: n = 12). A significantly lower diabetes incidence was observed in OM-85–treated CD1d\(^{-/-}\) NOD mice compared with untreated CD1d\(^{-/-}\) NOD mice (cumulative analysis P = 0.032). A trend toward lower diabetes incidence in OM-85–treated CD1d\(^{-/-}\) NOD mice versus OM-85–treated wild-type NOD mice was also observed. Experimental groups included 5–12 mice.

production in a TLR-dependent fashion was examined. The experimental model consisted of incubating primary bone marrow dendritic cells from C57BL/6 or genetically TLR- or MyD88-deficient mice with different concentrations of OM-85. As shown in Fig. 5, OM-85 stimulated the production of IL-12 in vitro. This response was not observed after stimulation of dendritic cells obtained from MyD88\(^{-/-}\) mice. When using dendritic cells from TLR-4\(^{-/-}\) and TLR-2\(^{-/-}\) mice, total or partial inhibition of IL-12 production was observed, respectively (Fig. 5). These results show that in dendritic cells OM-85 signals exclusively via the MyD88 pathway, at least partially by TLR-2 and -4.

OM-85 modulates TGF-\(\beta\) production via the TLR-MyD88 pathway. We next tested the ability of OM-85 to stimulate the in vitro production of IL-4, IL-10, IFN-\(\gamma\), and TGF-\(\beta\) using murine splenocytes. Importantly, OM-85 stimulation induced very high production of TGF-\(\beta\) (which was maximal already at 1 \(\mu\)g/ml in wild-type mice), high production of IL-10 (from 100 \(\mu\)g/ml), and moderate production of IFN-\(\gamma\) (from 100 \(\mu\)g/ml), whereas IL-4 production was undetectable (Fig. 6). Interestingly, TGF-\(\beta\) and IL-10 production were abolished in MyD88\(^{-/-}\) mice (Fig.
6). TGF-β production was also completely abolished in TLR-4−/− mice and partially in TLR-2−/− and TLR-4−/− mice compared with wild-type mice. The presence of TLR-4 agonists in OM-85, as suggested by these results, prompted us to examine the content of lipopolysaccharide in OM-85, using a limulus amebocyte assay. The level of lipopolysaccharide was found to be lower than that required for stimulating mononuclear phagocytes (0.1 pg of lipopolysaccharide per milligram of OM-85). One may thus assume that such traces of lipopolysaccharide cannot account for the observed TLR-4–mediated stimulatory capacity of OM-85.

**DISCUSSION**

The mechanisms of the protective effect of infections on type 1 diabetes are still elusive. Limited data are available in models based on infections in autoimmunity-prone mouse strains (2). Simplified approaches using bacterial components or viral proteins are difficult because there is no indication of the nature of the molecules responsible for the protection. It was thus of particular interest to use a bacterial extract that would putatively contain the various components participating in bacteria-mediated protection, whose handling is easier than live infections. In this vein, we selected OM-85, a bacterial extract prepared from eight bacterial species frequently responsible for respiratory tract infections. It has been reported earlier that another bacterial extract, OM-89, can afford protection in animals and/or humans against autoimmune diseases such as rheumatoid arthritis (20–21), lupus erythematosus (22), spondyloarthopathies (23), and diabetes (24–25). The product tested in the present study (OM-85) is of particular pertinence because it has extensively and safely been used in children suffering from repeated upper respiratory tract infections (26–27), thus paving the road to investigate its preventive potential in children prone to developing type 1 diabetes.

Results obtained in this study first show that OM-85 can prevent or delay type 1 diabetes onset when administered intraperitoneally at a low dosage. The product can also afford protection when given orally, but it must then be administered at a higher dosage. Importantly, the effect is optimal when the treatment is started early (3 or 6 weeks of age), but some protection is still achieved when the...
treatment is started at 10 weeks of age. Such kinetics are similar to those described for CFA (13). Importantly, two proinflammatory adjuvant-like yet nonimmunogenic compounds, namely, IFA and MDP (10–12,16–18), did not confer protection from diabetes, as opposed to OM-85 bacterial extract. However, currently, one cannot exclude that some proinflammatory compounds included in OM-85 actively participate in its protective effect in this model.

We examined the role of T-helper type 2 cytokines, namely IL-4 and -10, in the protective effect of OM-85. No responsibility of IL-4 or -10 could be evidenced by using genetically deficient mice for IL-4 and a neutralizing monoclonal antibody for IL-10 receptor. It is interesting to note that in the particular setting of newborn rats, OM-85 increased the production of IFN-γ and decreased that of IL-4 by spleen cells (28). When a neutralizing anti–TGF-β antibody was administered immediately after OM-85, a major role was evidenced for TGF-β because the protective effect of the drug was essentially lost. It is not clear, however, which cells produce TGF-β in this context and what its cellular target is. TGF-β could be produced by regulatory T-cells or induce their differentiation, as suggested in other models (29–33). Our in vitro studies show that OM-85 stimulates dendritic cells and induces very high levels of TGF-β production in a TLR-dependent fashion by splenocytes (TGF-β production was not observed using splenocytes derived from TLR-4−/− or MyD88−/− mice). This TLR-mediated effect of OM-85 may also involve TLR-2, although TLR-2−/− mouse splenocytes are still at least partly sensitive to OM-85. Other cytokines are produced on OM-85 stimulation, such as IL-12 and -10, which could open other perspectives. In any event, it is interesting to mention our unpublished recent results showing that various chemically defined TLR agonists afford type 1 diabetes protection in the same manner as OM-85: Pam3Cys and P40 for TLR-2, poly(I:C) for TLR-3, and lipopolysaccharide for TLR-4. As far as OM-85 is concerned, lipopolysaccharide is probably not the moiety involved in TLR-4 stimulation because it is not found in the extract, even at a very low concentration.

We recently reported a critical role for CD1d-dependent NKT cells in exacerbated T-helper type 2-mediated airway inflammation and hyperresponsiveness (34) and in autoimmune diabetes–prone NOD mice (35–36). The data presented in this article confirm that NKT cells are implicated in type 1 diabetes. One should mention, however, that in our present study, both mouse strains (CD1d−/− and wild type) showed a similar type 1 diabetes incidence, a finding in clear contrast with another report (37), but in the line of that of Van Kaer (38), who provided us with the mice used here. If NKT cells are indeed involved in the OM-85–mediated protection, it will be important to determine which component within OM-85 stimulates NKT cells and what mechanisms mediate NKT cell involvement. IL-4, as suggested by Oki et al. (39) and Mi et al. (40), and IL-10, as suggested by Mi et al. (41), could be incriminated, even though we have a priori excluded these two cytokines as major protagonists in our type 1 diabetes model. In any case, the role of NKT cells in type 1 diabetes protection is only partial because CD1d−/− NOD mice still show some sensitivity to OM-85.

An implication of effector cells is possible though unlikely in our model because treating NOD SCID mice with OM-85 after transfer of diabeticogenic T-cells does not delay diabetes onset (data not shown). Note, however, that other mechanisms cannot be excluded. Thus, it has been shown that Bacille Calmette-Guerin, CFA (42), or the lymphocytic choriomeningitis virus (43) may induce protection through IFN-γ− and TNF-α–mediated T-cell apoptosis.

To conclude, these studies bring new insight into the cellular and molecular mechanisms underlying the type 1 diabetes protection afforded by OM-85. Our data incriminate TGF-β and NKT cells, possibly through TLR stimulation. One possibility linking TGF-β and the antidiabetic effect observed in our model could be that the OM-85–induced secretion of TGF-β (as reported here) would in turn induce the production of regulatory FoxP3+ cells from CD4+CD25+ precursors, as recently suggested (44). Further studies are needed to elucidate this point. Other mechanisms are of course not excluded. One may assume that the complexity of the OM-85 molecular composition contributes to the multifactorial nature of its mode of action. It would be interesting to analyze the action of individual components of the extract. On the other hand, one must take into account the fact that OM-85 is used clinically and that the simultaneous presence of several pharmacologically active components represents an interesting correlation to the overall effect of bacterial infections.

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