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Streptococcal wall component OK432 restores sensitivity of non-obese diabetic (NOD) thymocytes to apoptotic signals

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Abstract

Aims/hypothesis. The streptococcal wall component, OK432, prevents diabetes in NOD mice and BB rats by elimination of effector cells. Based on the knowledge of a link between autoimmunity and resistance of immune cells to elimination by apoptosis, we investigated whether OK432 treatment restored the sensitivity of NOD lymphocytes to apoptotic signals centrally (thymus) or peripherally (spleen) or both and we examined the pathways for the enhanced apoptosis rate.
Methods. We treated NOD mice with OK432 (0.1 mg/kg i.p. weekly from 21 to 70 days). Apoptosis was measured by TUNEL 16 h after cyclophosphamide (70 mg/kg) and 24 h after dexamethasone (0.2 mg/mouse). Real time quantitative RT-PCR was used to investigate changes in gene expression.
Results. Thymocyte apoptosis levels after cyclophosphamide were restored by OK432 treatment to levels observed in C57BL/6 mice: in NOD males apoptosis increased from $8 \pm 1\%$ to $18 \pm 5\%$ ($p < 0.05$) compared with $20 \pm 4\%$ in C57BL/6 males, and in NOD females from $6 \pm 2\%$ to $11 \pm 2\%$ ($p < 0.05$) compared with $12 \pm 2\%$ in C57BL/6 females. The dexamethasone-induced thymocyte apoptosis rate was equally restored by OK432 treatment ($58 \pm 4\%$ vs $41 \pm 3\%$ in control males ($p < 0.0005$) and $39 \pm 5\%$ vs $26 \pm 3\%$ in control females ($p < 0.05$)]. No change in apoptosis levels was on the contrary observed in splenocytes after OK432 treatment. By RT-PCR analysis of a panel of apoptosis-related genes in thymocytes we showed a down-regulation of anti-apoptotic Bcl-xL and c-myc by OK432 treatment.
Conclusions/interpretation. Our data suggest that OK432 prevents diabetes in NOD mice by better elimination of effector cells through increased sensitivity to apoptotic signals centrally in the thymus. [Diabetologia (2000) 43: 1302–1308]

Keywords Type I diabetes, apoptosis, NOD mice, thymus, OK432.

Type I (insulin-dependent) diabetes mellitus is an autoimmune disease characterised by destruction of pancreatic beta cells. In NOD mice, a widely used animal model of Type I diabetes, the progression to this destruction is inhibited by immunosuppressants but also by immunostimulators such as complete Freund's adjuvant (CFA) or bacille Calmette-Guérin (BCG) [1, 2]. In the latter treatments, the generation of suppressor cells is considered to be the mechanism of protection, because injection of cyclophosphamide, an agent believed to eliminate suppressor cells [3–5], can undo the protection from diabetes.

The streptococcal wall component, OK432, is a biological response modifier and has been clinically used in Japan for immunotherapy of cancer for more than 20 years without side effects [6–10]. It has also been reported to be effective in the treatment of autoimmune diseases, especially in animal models of Type I diabe-

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Abbreviations: NOD, Non-obese diabetic; BCG, bacillus Calmette-Guerin; CFA, complete Freund's adjuvant; TUNEL, Terminal deoxynucleotidyl transferase (TdT) mediated fluorescein isothioryanate conjugate-2' deoxyuridine 5'-triphosphate (FITC-dUTP) nick end labelling reaction.

tes [11, 12]; treating NOD mice and BB rats with OK432 resulted in a protection from insulitis and from the development of overt diabetes. Transfer/co-transfer experiments and injection of cyclophosphamide showed that OK432 exerts its function through elimination of diabetogenic effector cells [13].

The aim of our study was to further explore the mechanism of this effector cell elimination by OK432. In the first part of the study we wanted to confirm the protective role of OK432 in a model of cyclophosphamide-induced diabetes in NOD mice. As a link between diabetes in NOD mice and a lymphocyte resistance to apoptosis has been shown [14–18], we hypothesised in the second part of this study that OK432 restores the susceptibility of NOD lymphocytes to go into apoptosis. To test this hypothesis we examined the apoptosis percentage in NOD thymus and spleen cells after apoptotic stimuli such as dexamethasone and cyclophosphamide. Finally, in search of the molecular mechanism underlying the change in apoptosis sensitivity, we studied the expression of different pro-apoptotic and anti-apoptotic markers in thymocytes of control NOD mice and NOD mice treated with OK432 to examine if a change in the pattern of gene expression correlates with a decrease in apoptosis sensitivity.

Materials and methods

Animals. Inbred NOD mice in our animal facility since 1990 were kept under conventional conditions. The principals of laboratory animal care (NIH publication no. 85–23, revised 1985) were followed and all experiments were approved by the local animal ethics committee. The incidence of spontaneous diabetes in stock mice at the age of 200 days at the time of the study was 72 % in females and 23 % in males. We purchased C57BL/6 mice from Charles River (Wiga, Sulzfeld, Germany) and used these as the control strain.

Treatment regimen. Picibanil (Chugai Pharmaceutical Co., Tokyo, Japan) or OK432 is a heat-treated and penicillin-treated lyophilised preparation of the Su strain of *Streptococcus pyogenes* A3. It was suspended in PBS for injection and given intraperitoneally at 0.1 mg/kg once a week, starting at 21 days of age, the time of weaning, until 70 days of age. We used PBS vehicle as sham treatment.

Diabetes precipitation and evaluation. In the first experiment and control NOD mice and those treated with OK432 received a single cyclophosphamide injection (Endoxan, ASTA Medica, Brussel, Belgium) of 200 mg/kg at 70 days of age. To evaluate diabetes, mice were weighed weekly and glucosuria was tested 3 times a week using Clinistix (Bayer Diagnostics, Bastingstoke, UK). When glucosuria was positive, glycaemia was measured in tail vein blood (Glucocard, Menarini, Florence, Italy) and diabetes was diagnosed in mice with a glycaemia above 13.8 mm/dl on 2 consecutive days. At the time of diabetes diagnosis mice were killed by ether inhalation and cervical dislocation. In the case of normoglycaemia mice were followed for diabetes until 40 days after cyclophosphamide injection and then killed.

Apoptosis induction and detection. Apoptosis was induced by intraperitoneal injection of dexamethasone (Aacidexam, N. V. Organon, Oss, The Netherlands, 0.2 mg/mouse) or cyclophosphamide (70 mg/kg) at 70 days of age in OK432-treated and control NOD mice as well as in C57BL/6 mice. Apoptosis was measured separately in male and female mice. To evaluate background apoptosis levels, apoptosis was measured in mice from each group without dexamethasone or cyclophosphamide injection.

Apoptosis was detected using the terminal deoxynucleotidyl transferase-(Tdt) mediated fluorescein isothiocyanate conjugate-2'-deoxyuridine 5'-triphospate (FITC-dUTP) nick end labelling reaction (TUNEL) method. Briefly, OK432-treated NOD mice, control NOD mice and C57BL/6 mice were killed by ether inhalation and bled to induce hypovolaemia. Spleens and thymi were isolated before, 16 h after cyclophosphamide or 24 h after dexamethasone injection. These time points result from a kinetic study, where differences in apoptosis sensitivity were most pronounced after 16 h for cyclophosphamide and after 24 h for dexamethasone (C. Mathieu, unpublished data). The organs were kept on ice and then gently teased apart and pressed through a steel mesh. Cells were counted and afterwards fixed for 30 min at room temperature in 2 % paraformalde hyde in PBS, permeabilised with 0.1 % saponin in PBS for 2 min and incubated for 1 h at 37 °C with TdT and FITC-conjugated dUTP (Boehringer Mannheim, Brussel, Belgium). Cells were then washed and analysed with a FACScan (Becton Dickinson, Erembodegem, Belgium). Apoptosis is expressed as percentage TUNEL positive cells.

Thymic apoptotic markers. At 70 days of age thymi were isolated from OK432-treated and control NOD mice after mice were ether anaesthetised and bled to induce hypovolaemia. Total RNA was immediately extracted using TRIzol reagent (Gibco BRL Life Technologies, Merelbeke, Belgium). A constant amount of 2.5 μg of target RNA was reverse transcribed with Superscript II reverse transcriptase (Gibco BRL Life Technologies) at 42 °C for 80 min in the presence of oligo $d(T)_{16}$ (Perkin Elmer/Applied Biosystems, Foster City, Calif., USA). For Fas, Fas Ligand, Bcl-2, Bax, Bcl-xL, IL-15, c-fos, c-jun, c-myc, TNFα and β-actin (housekeeping gene) real time quantitative RT-PCR was done as described previously [19]. Table 1 shows the sequences of the self designed primers and probes. Briefly, PCR reactions were done in the ABI Prism 7700 sequence Detector (Perkin Elmer/Applied Biosystems). The assay uses the 5'nuclease activity of *Taq* polymerase to cleave a non-extendible dual labelled fluorogenic hybridisation probe during the extension phase of the PCR reaction. One fluorescent dye serves as a reporter (FAM) and its emission spectra is quenched by the second fluorescent dye, TAMRA. The nuclease degradation of the hybridisation probe releases the quenching of FAM, resulting in an increase in peak fluorescent emission at 518 nm. Measurement of fluorescent spectra of all 96 wells of the thermal cycler is done by a sequence detector (ABI prism) continuously during PCR amplification. Therefore, amplification and detection are done in one single step. Each PCR reaction was done in a total volume of 25 μl, containing 0.5 μl of cDNA, 2.5 μl 10 × *Taq*Man buffer A, 3 to 7 mmol/l $MgCl_2$, 200 μmol/l deoxy-adenosine 5'-triphosphate (dATP), deoxy-cytosine 5'-triphosphate (dCTP), deoxy-guanosine 5'-triphosphate (dGTP) and 400 μmol/l deoxy-thymidine 5'-triphosphate (dTTP), 100 to 300 nmol/l of each self-designed primer and 0.625 units AmpliTaq Gold (Perkin Elmer/Applied Biosystems). Each reaction also contained 100 to 200 nmol/l of the corresponding detection probe. Each PCR reaction was done in duplicate, using the following conditions: 10 min at 94 °C, followed by a total of 40 two-tem-

Table 1. Primer and probe sequences

	Forward primer	Reverse primer	Probe (FAM-TAMRA)	Amplicon length (base pairs)
β-actin	AGAGGGAAATCGTGCGT-GAC	CAATAGTGAT-GACCTGGCCGT	CACTGCCG-CATCCTCTTCCTCCC	148
Fas	CTGCGATGAAGAG-CATGGTTT	CCATAGGCGATTTCTGGGAC	TGCGATTCTCCTGGCTGT-GAACACTG	208
Fas L	AAGAAGGACCACAACA-CAAATCTG	CCCTGTTAAATGGGCCA-CACT	TGCAGAAGGAACTGGCA-GAACTCCG	234
Bcl-2	CTTAGAAAATACAG-CATTGCGGAG	GGATGTGCTTTGCATTCTTGG	TTCCTGCATCTCATGC-CAACGGG	194
Bcl x-L	CACTGTGCGTGGAAAGCGTA	AAAGTGTCCCAGCCGCC	CAAGGAGATGCAGG-TATTGGTGAGTCGG	127
Bax	GTTTCATCCAGGATCGAG-CAG	CCCCAGTTGAAGTTGCCAC-CATC	AGCTGAGC-GAGTGTCTCCGGCG	238
TNFα	CATCTTCTCAAAATTCGAGT-GACAA	TGGGAGTAGACAAGGTA-CAACCC	CACGTCGTAGCAAACCAC-CAAGTGGA	175
IL-15	CATCCATCTCGTGC-TACTTGTGTT	CATCTATC-CAGTTGGCCTCTGTTT	AGGGAGACCTACACTGACA-CAGCCCAAAA	126
c-fos	CTCCTTCTCCAGCATGGGC	GGGATAAAGTTGGCACTA-GAGACG	TCAACACACAG-GACTTTTGCGCAGATCT	81
c-jun	CCTGTCCCCTATCGACATGG	CTTTTCCGGCACTTGGAGG	TCCTCATGCGCTTCCTCTCTG-CCT	93
c-myc	TGAGCCCCTAGTGCTGCAT	ACGCCGACTCCGACCTCTT	CTTCTTGCTCTTCTTCA-GAGTCGCTGCTG	137

perature cycles (15 s at 94 °C and 1 min at 60 °C). Quantification of input cDNA from the unknown samples was done by including a standard curve. For the generation of these standard curves, plasmid clones containing a partial cDNA sequence for each of the genes of interest were constructed. Briefly, total RNA was extracted from thymi, reverse transcribed and PCR was done using the same primers as desribed in Table 1. The amplicons were cloned into a pGEM-Teasy plasmid vector (Promega, Leiden, The Netherlands). The length of the amplicons was confirmed by restriction analysis. Serial dilutions from the plasmid clones were used as standard curves, each containing a known amount of template copy number. A normalisation to β-actin (housekeeping gene) was done for each sample.

Statistical analysis. For diabetes incidence the chi-squared test was used. For the TUNEL and RT-PCR data within sex groups, the unpaired two-tailed Student *t* test was used. Significance was defined at the 0.05 level. For the TUNEL and RT-PCR data between sex groups, the ANOVA and Kruskal Wallis Multiple Comparison *Z* value test were used. Data are expressed as means ± SEM.

Results

OK432 treatment protects NOD mice from cyclophosphamide-induced diabetes. Given intraperitoneally, OK432, at a dose of 0.1 mg/kg every week from the time of weaning until 70 days of age, statistically significantly reduced diabetes incidence after cyclophosphamide injection. Of the control male NOD mice 67 % (6/9 animals) developed diabetes, whereas only 11 % (1/9 animals) of the male OK432-treated mice developed diabetes 40 days after cyclophosphamide injection ($p < 0.05$, Fig. 1). This decrease in diabetes incidence was even more pronounced in the female group of mice, where 83 % (10/12 animals) of the control mice developed diabetes after cyclophosphamide, in contrast to 25 % (3/12 animals) of the OK432-treated mice ($p < 0.005$, Fig. 1). No local or general side effects were seen during or after OK432 treatment.

Restored apoptosis sensitivity in thymocytes of OK432-treated NOD mice after dexamethasone injection. As already described in previous studies, NOD lymphocytes are highly resistant to apoptosis compared with mouse strains that are not autoimmune-prone such as C57BL/6 mice [14–18]. Moreover, female lymphocytes (NOD as well as C57BL/6) are more resistant to apoptosis than their male counterparts [20]. After dexamethasone injection, apoptosis of thymocytes significantly increased from 41 ± 3 % in the control male NOD group to 58 ± 4 % in the OK432-treated group ($p < 0.0005$, Fig. 2 a). Similarly in the OK432-treated female group, the apoptosis level rose from 26 ± 3 % to 39 ± 5 % ($p < 0.05$, Fig. 2). When measuring background apoptosis levels with the TUNEL method, no significant differences were seen between the different experimental groups (data not shown). Furthermore, we confirmed the previously observed sex difference in apoptosis sensi-

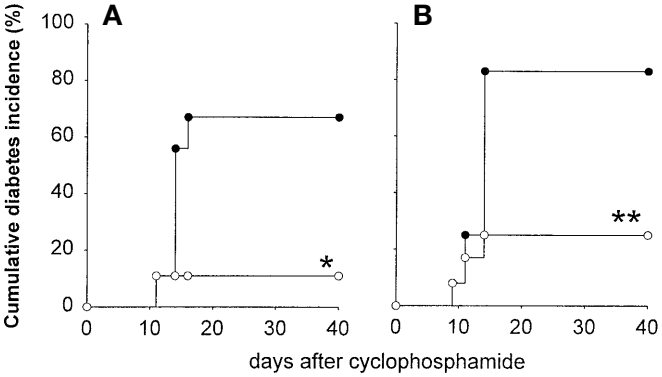

Fig. 1 A, B. Cumulative diabetes incidence in OK432-treated (○) and control NOD mice (●) after cyclophosphamide injection (200 mg/kg) at 70 days of age. Male NOD mice are represented in **A** ($n = 9$ treated and $n = 9$ control mice) and female NOD mice are represented in **B** ($n = 12$ treated and $n = 12$ control mice). Mice were followed for diabetes until 40 days after cyclophosphamide injection. * $p < 0.05$ vs male NOD mice, ** $p < 0.005$ vs female NOD mice

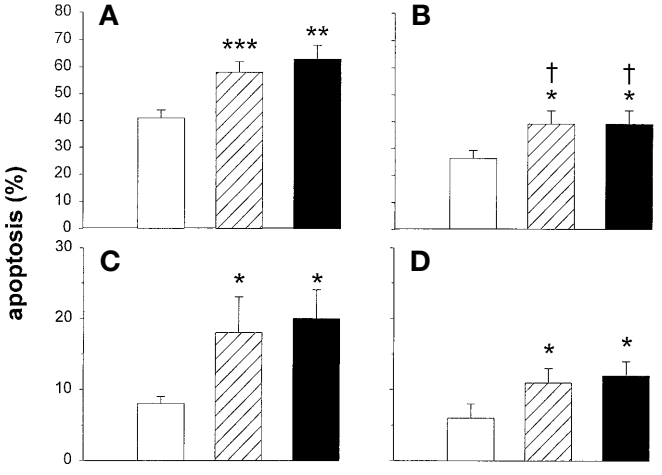

Fig. 2 A, B. Dexamethasone induced apoptosis in thymocytes of male (**A**) and female (**B**) NOD mice, OK432-treated NOD mice and C57BL/6 control mice. Apoptosis was measured 24 h after dexamethasone injection and is expressed as percentage TUNEL positive cells. **C, D** Cyclophosphamide induced apoptosis in thymocytes of male (**C**) and female (**D**) NOD mice, OK432-treated NOD mice and C57BL/6 control mice. Apoptosis was measured 16 h after cyclophosphamide injection and is expressed as percentage TUNEL positive cells. Each group represents the means ± SEM of 6–11 mice. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ vs NOD mice of the corresponding sex. † $p < 0.05$ vs corresponding group of the opposite sex. ☐ NOD; ▨ NOD-OK432; ■ C57BL/6

tivity of thymocytes in the female groups compared with the male groups: 26 ± 3 % compared with 41 ± 3 % (p = NS) in the female and male control NOD groups, respectively; 39 ± 5 % compared with 58 ± 4 % ($p < 0.05$) in the female and male OK432-treated NOD groups and 39 ± 5 % compared with 63 ± 5 % ($p < 0.05$) in the female and male C57BL/6

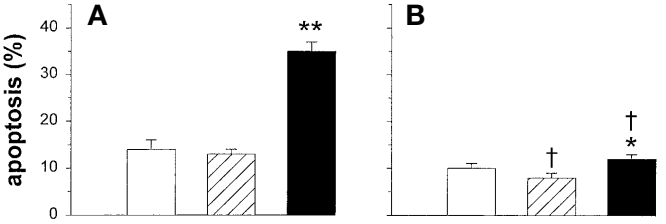

Fig. 3 A, B. Cyclophosphamide-induced apoptosis in splenocytes of male (**A**) and female (**B**) NOD mice, OK432-treated NOD mice and C57BL/6 control mice. Apoptosis was measured 16 h after cyclophosphamide injection and is expressed as percentage TUNEL positive cells. Each group represents the means ± SEM of 6–15 mice. * $p < 0.05$, ** $p < 0.0005$ vs NOD mice of the corresponding sex. † $p < 0.05$ vs corresponding group of the opposite sex. ☐ NOD; ▨ NOD-OK432; ■ C57BL/6

groups. Thus OK432 restored apoptosis levels in NOD male and female thymocytes to those observed in C57BL/6 male and female thymocytes, maintaining the sex difference.

Restored apoptosis sensitivity in thymocytes but not in splenocytes of OK432-treated NOD mice after cyclophosphamide injection. We found 8 ± 1 % apoptotic thymocytes in control male NOD mice compared with 20 ± 4 % in C57BL/6 male mice after cyclophosphamide injection ($p < 0.05$). In control female NOD thymocytes an apoptosis rate of 6 ± 2 % was seen, whereas female C57BL/6 mice had 12 ± 2 % apoptotic thymocytes ($p < 0.05$). Treatment with OK432 significantly increased the apoptosis rate in male (18 ± 5 %, $p < 0.05$) as well as in female NOD thymocytes (11 ± 2 %, $p < 0.05$) to that observed in C57BL/6 thymocytes (Fig. 2). In the splenocytes (Fig. 3) on the contrary, no significant differences in apoptosis could be seen between the OK432-treated and control groups [13 ± 1 % in the OK432-treated vs 14 ± 2 % in the control male group (p = NS) and 8 ± 1 % in the OK432-treated vs 10 ± 1 % in the control female group (p = NS)), whereas in spleens from male and female C57BL/6 mice apoptosis was 35 ± 2 % ($p < 0.0005$ vs control male NOD mice) and 13 ± 1 % ($p < 0.05$ vs control female NOD mice), respectively.

Down-regulated mRNA expression of Bcl-xL and c-myc in thymocytes of OK432-treated NOD mice. Using real time quantitative RT-PCR, thymic mRNA expression of several pro/anti-apoptotic markers was measured in thymi from control and OK432-treated NOD mice at 70 days of age. For Fas, Fas Ligand, IL-15, Bcl-2, Bax, c-jun, c-fos and TNFα, no differences in mRNA expression were observed between control and OK432-treated NOD mice (data not shown). In contrast, a very clear thymic down-regulation of Bcl-xL mRNA by OK432 was seen in male treated compared with male control

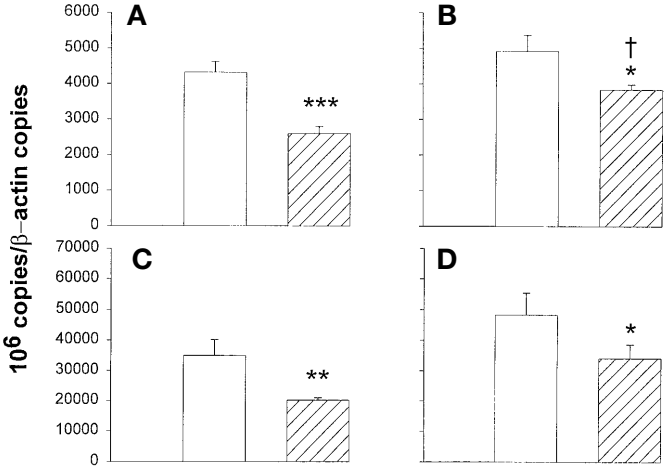

Fig. 4 A, B. Bcl-xL relative to β-actin mRNA expression in male (**A**) and female (**B**) thymocytes of NOD mice and OK432-treated NOD mice at 70 days of age. **C, D** c-myc relative to β-actin mRNA expression in male (**C**) and female (**D**) thymocytes of NOD mice and OK432-treated NOD mice at 70 days of age. Each bar represents the means ± SEM of 5–12 mice. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$ vs NOD mice of the corresponding sex. $^{\dagger}p < 0.05$ vs male OK432-treated NOD mice. ☐ NOD; ▨ NOD-OK432

NOD mice (2598 ± 596 vs 4313 ± 684 × 10^6 copies/β-actin copies, $p < 0.0005$) (Fig. 4). Similarly in the female group (Fig. 4), a significant Bcl-xL down-regulation was observed in the treated compared to the control group ($p < 0.05$). The Bcl-xL down-regulation was present in both sexes but females maintained higher thymic levels after OK432 treatment ($p < 0.05$ vs OK432-treated male NOD mice).

Thymic mRNA expression of c-myc, a proto-oncogene and not a member of the Bcl-2 superfamily, was also down-regulated after OK432 treatment in male NOD mice (20100 ± 2770 vs 34871 ± 11476 × 10^6 copies/β-actin copies in male control NOD mice, $p < 0.005$) (Fig. 4). Similarly, a decrease of c-myc mRNA expression was observed in female thymi ($p < 0.05$, Fig. 4).

Discussion

For 20 years OK432, a streptococcal wall component, has been studied and used for adjuvant cancer therapy in Japan with clinical improvement and prolongation of survival in cancer patients [6–10]. To exert tumour destruction, OK432 seems to have a cell regulatory activity through its ability to activate T lymphocytes, macrophages and enhance NK cell activity [21–24]. Therapy with OK432 can also prevent insulitis, spontaneous and cyclophosphamide-induced diabetes in NOD mice and BB rats [11–13]. It is a strong polycytokine inducer, especially of TNFα, lymphotoxin, IL-1α, IL-2, IL-6, IL-12 and IFNγ [10].

We first confirmed data previously reported by others [13], namely that OK432 treatment of young non-diabetic NOD mice protects against diabetes accelerated by cyclophosphamide, an agent believed to eliminate suppressor cells [3–5]. Because protection against diabetes by OK432 cannot be broken by cyclophosphamide, this protection is probably mediated through an elimination of effector cells. These data are supported by transfer experiments, where splenocytes of OK432-treated NOD mice hardly transferred diabetes [13].

To explain the mechanism of effector cell elimination by OK432, in the second part of this study we examined a possible link between OK432 and apoptosis sensitivity in the immune system. Our group previously showed that 1,25-dihydroxyvitamin D_3 ($1\alpha,25(OH)_2D_3$), the active form of vitamin D, prevents insulitis and diabetes in NOD mice by inducing suppressor cells but also by eliminating effector cells (injection of cyclophosphamide, transfer/cotransfer experiments), most probably through restored apoptosis sensitivity of immunocytes centrally and peripherally [25–27]. Similarly for OK432, we found a clear increase in the apoptosis sensitivity of thymocytes after apoptosis inducing signals such as dexamethasone or cyclophosphamide. Treatment with OK432 increased the apoptosis percentage of NOD thymocytes after apoptotic signals to normal levels, as observed in C57BL/6 mice. Notably, in contrast to its effects in the central immune system, there was no enhancement of apoptosis sensitivity in the peripheral immune system. It can be hypothesised that OK432 breaks central lymphocyte resistance to apoptosis and contributes to better thymic negative selection of diabetogenic immune cells, thus preventing them from reaching the periphery as effector cells. This is in accordance with previously reported in vivo data [13], where OK432 was shown to be far more efficient when given at early age (from 4 to 9 weeks of age) than at older age (from 10 to 15 weeks of age), when effector cells are already present in the peripheral immune system. We therefore suggest that OK432 eliminates effector cells but this is only achieved in the central and not in the peripheral immune system.

Further experiments to understand the molecular mechanisms of restored apoptosis sensitivity in the thymus by OK432 showed a clear down-regulation of anti-apoptotic Bcl-xL, a member of the Bcl-2 superfamily and of oncogenic c-myc mRNA after OK432 treatment. A crucial role for Bcl-xL as a survival signal in immature CD4 + CD8 + thymocytes has been suggested by different groups [28–30]. It is highly expressed in CD4 + CD8 + thymocytes but there is a relative deficiency of Bcl-2 compared to other thymocyte maturation stages [29, 30]. Thus Bcl-xL down-regulation plays an important part in sharpening sensitivity of immature thymocytes to apoptotic signals. Mice deficient in Bcl-x have a mas-

sive cell death of immature, not mature, haematopoietic cells and neurons, again suggesting an important role for Bcl-xL in the immature stage of cells [31]. It has also been found that Bcl-xL is upregulated in activated NOD lymphocytes and this was most pronounced 24 h after IL2-deprivation [32]. According to our results this could mean that the apoptosis resistance of NOD lymphocytes is the result of a Bcl-xL dysregulation. It is, however, more likely that a number of dysregulations of the thymocyte death/survival system should exist before autoimmunity occurs. The proto-oncogene c-myc, for which we also found a thymic down-regulation after OK432 treatment, probably is a survival factor in immature thymocytes [33, 34]. Again nuclear factor $\varkappa$B (NF-$\varkappa$B) is involved in this pathway. Thus c-myc down-regulation by OK432, together with other changes such as Bcl-x L down-regulation, eventually could favour apoptosis of autoreactive cells. It seemed that Fas, Fas Ligand, Bax, Bcl-2 and IL-15 were not involved in the central apoptosis restorative effect of OK432. Also for TNFα, considered as one of the defective mediators especially in female NOD mice and being up-regulated after OK432 treatment [35–39], no significant changes were observed at the thymic mRNA expression between the different experimental groups. We could observe neither a thymic TNFα up-regulation in the OK432-treated groups nor a difference in thymic TNFα production between the sexes. It has also been reported that c-fos and c-jun have a low expression in female NOD thymocytes and an upregulation was found after treatment with human TNFα, human lymphotoxin and human IL-2 [40]. We could not confirm a c-fos-mRNA up-regulation or a c-jun mRNA up-regulation, although we used OK432, a strong TNFα inducer.

Finally, we confirmed that sexual dimorphism in diabetes incidence in NOD mice is paralleled by a dimorphism in resistance to apoptotic signals in thymocytes, as was previously shown by our group [20]. We also found higher expressions of Bcl-xL and c-myc in female mice. This possibly correlates with the lower apoptosis sensitivity in female NOD mice. Sex hormones might be one of the reasons of sexual dimorphism in apoptosis, possibly through different Bcl-xL and c-myc expression.

We confirmed that OK432 prevents cyclophosphamide-induced diabetes in NOD mice. We propose increased apoptosis sensitivity centrally in the thymus as a mechanism of effector cell elimination by OK432. Down-regulation of Bcl-xL and c-myc seems to be a contributing factor in this central apoptosis restoration.

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