Diabetologia (1998) 41: 1298–1303

Diabetologia

Soluble forms of intercellular adhesion molecule-1 inhibit insulitis and onset of autoimmune diabetes

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Summary Increased concentration of circulating adhesion molecules in human serum have been described in different immune-mediated diseases. Recently, we proposed an immunomodulatory function of soluble forms of the intercellular adhesion molecule-1 (ICAM-1) during the pathogenesis of human Type I (insulin-dependent) diabetes mellitus. To test this hypothesis in nonobese diabetic (NOD) mice, a spontaneous animal model for human Type I diabetes, two recombinant forms of soluble murine ICAM-1 were generated, one monomeric soluble ICAM-1 containing all five extracellular Ig-like domains of ICAM-1 (rICAM-1) and one dimeric protein with the N-terminal extracellular domains fused to the constant regions of murine IgG2a (rICAM-1-Ig). Beginning at age 35 days prediabetic NOD mice received i.p. injections of 5 μg recombinant ICAM-1-proteins three times a week for 4.5 months. At day 170 diabetes development was reduced ($p < 0.001$) in NOD mice receiving rICAM-1 (8%) or rICAM-1-Ig (8%) treatment in comparison with sham treated animals (45%). After termination of therapy animals treated with multimeric rICAM-1-Ig were protected longer than animals treated with rICAM-1. Prevention of diabetes was associated with decreased infiltration of pancreatic islets by mononuclear cells. A selective downregulation of Th1-type cytokine expression was observed in a second set of experiments in which diabetes development was synchronised by cyclophosphamide. These data support the hypothesis that circulating forms of adhesion molecules have an immunomodulatory function and can intervene in islet inflammation. [Diabetologia (1998) 41: 1298–1303]

Keywords Immunotherapy, in vivo animal models, adhesion molecules.

Type I (insulin-dependent) diabetes mellitus is preceded by a prodromal phase which can be diagnosed by the presence of several islet specific autoantibodies in human serum [1, 2].

Received: 21 April 1998 and in final revised form: 17 July 1998

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Abbreviations: ICAM-1, Intercellular adhesion molecule-1; rICAM-1, recombinant ICAM-1; Th1/Th2, T-helper type $^1/_2$; NOD mouse, nonobese diabetic mouse; PCR, polymerase chain reaction; mICAM-1, murine ICAM-1; RT-PCR, reverse transcriptase-PCR; IFN-gamma, interferon-gamma; IL-4, Interleukin-4; MOI, multiplicity of infection.

Before and at onset of overt disease we previously observed raised serum concentrations of circulating intercellular adhesion molecule-1 (ICAM-1) and L-selectin when compared with healthy control subjects. Surprisingly, people with a high genetic risk of Type I diabetes (HLA-DR3 or -DR4 or both) but without any immunological signs (islet cell autoantibody negative) of developing Type I diabetes showed very high concentrations of circulating ICAM-1 and L-selectin [3]. We therefore suggested an immunoprotective function for circulating adhesion molecules counteracting the activation of autoimmune T-cells. In support of this hypothesis we reported that the reactivity of autoimmune T-cells from recent onset Type I diabetic patients in response to an islet specific autoantigen can be suppressed in vitro by recom-

binant soluble ICAM-1 (rICAM-1) in concentrations similar to increased serum concentrations of circulating ICAM-1 [4].

In addition to providing co-stimulatory signals in T-cell activation [5], adhesion molecules mediate the adhesion and triggering of leucocytes during emigration from circulation into lymph nodes or other tissue [6]. Hence, adhesion molecules have an important role in leucocyte accumulation and activation during organ inflammation [7]. Evidence of an important role of ICAM-1/LFA-1 interaction during the development of autoimmune (Type I) diabetes in the non-obese diabetic (NOD) mouse comes from studies with specific monoclonal antibodies in vivo [8, 9]. The NOD mouse model was therefore chosen to determine whether soluble ICAM-1 is able to interfere with the disease process in vivo. In this study we show that exogenous soluble ICAM-1 has a potent inhibitory effect on the development of autoimmune diabetes in NOD mice. A key event appears to be the downregulation of beta-cell destructive islet inflammation by selective suppression of T helper Type I immune responses.

Subjects, materials and methods

Construction of recombinant forms of murine ICAM-1. The cDNA for soluble murine ICAM-1 was generated by polymerase chain reactions (PCR). The pCDM8 [10] expression vector containing the complete murine ICAM-1 (mICAM-1) was used as template with a pCDM8 5′ primer (5′-CTTCTAGAGATCCCTCGACC-3′) and a 3′ primer (5′-TTTTCTAGATCAGTTATTTTGAGAGTGGTACAGTAC-3′) that encodes the end of domain 5 including the codon for N-460, a stop codon and a Xba I restriction site. The murine ICAM-1-Ig chimer was generated by two PCR products encoding the first two extracellular domains of mICAM-1, the hinge and the constant regions C_H2 and C_H3 of murine IgG2a. The mICAM-1 PCR fragment was generated by the pCDM8 5′ primer and a 3′ primer (5′-TTCTCGAGCCTCACCAAGATCGAAAGTCCGGAGGCTCC-3′) encoding the last 23 bases of the second extracellular domain, a 5′ donor sequence and a Xho I restriction site. The murine IgG2a PCR fragment was amplified by a 5′ primer (5′-TTTCTCGAGGGAGTAGAGGTTCACAAGTGATTAG-3′) encoding a Xho I site, a 3′ acceptor sequence and 18 bases encoding the first amino acids of the IgG2a hinge region. The 3′ primer (5′-TTTGCGGCCGCGACCT-GAGAGTTTTGTGGGTGCTG-3′) contained the coding region of the last 7 amino acids of the third constant domain of mIgG2a, a stop codon and a Not I restriction site. Both constructs were ligated into the pBlueBac2 transfer vector (Invitrogen, San Diego, Calif., USA) and recombinant viruses were generated by standard procedere using a transfection modul (AcMNPV Linear DNA Transfection Module, Invitrogen). Cloning of recombinant virus was done by plaque purification using dilution series of virus as described before [11]. Secretion of recombinant protein was monitored by a murine ICAM-1-ELISA (Pharmingen, Cambridge, Mass., USA). High virus titres were obtained by infection of Sf9 cells with recombinant virus at a multiplicity of infection (MOI) of 1. Virus titres of up to 1×10^9 pfu were recovered.

Production of recombinant forms of murine ICAM-1. Large scale production of recombinant ICAM-1 (rICAM-1) and ICAM-1-Ig (rICAM-1-Ig) was carried out by infecting Sf9 cells at a MOI of 10. Supernatants were harvested and recombinant proteins were purified by immunoaffinity chromatography on murine ICAM-1 monoclonal antibody YN1/1.7.4.-sepharose (Pharmacia, Uppsala, Sweden). Production and purification of YN1/1.7.4. was described earlier [12]. Recombinant proteins were eluted at high pH [13] and concentrations were calculated by measuring optical density at 280 nm. Extinction coefficients of 0.80 for ICAM-1 and 0.97 for ICAM-1-Ig were calculated using a peptide structure programme [14]. Purified proteins were subjected to SDS-PAGE electrophoresis under reducing and non-reducing conditions followed by silver staining. rICAM-1 yielded a single band at expected molecular weight at around 65 kDa (reduced and nonreduced), and rICAM-1-Ig at arround 60 kDa (reduced) or 120 kDa (nonreduced), suggesting that rICAM-1 is expressed as monomeric and rICAM-1-Ig as dimeric protein (data not shown).

Animal experiments. NOD mice (female NOD/Bom) were purchased from Bomholtgard Breeding Centre (Ry, Denmark) at 25 days of age and maintained in our animal facility under conventional conditions with standard diet and tap water ad libitum.

Animals were randomized in three groups of 36 NOD mice and treated with i.p. injections of rICAM-1, rICAM-1-Ig or vehicle (250 mmol/l Tris, 150 mmol/l NaCl, 70 mmol/l triethylamine, pH 7.5). Treatment was started at day 30 with 5 μg recombinant proteins three times a week until day 150, then 7.5 μg of the different proteins two times a week until day 170. From each group 5 animals were killed at day 132 for histological analysis of the pancreas, when 20% of the control group had developed Type I diabetes. Development of diabetes was monitored by daily urine glucose analysis, confirmed by blood glucose determination until day 260. Diabetes was defined by blood glucose concentration above 11 mmol/l on three consecutive days.

In a different set of experiments development of diabetes was synchronized and accelerated in NOD mice at 9 weeks with a single injection of cyclophosphamide (250 mg/kg i.p.) [15]. Three groups of 10 animals each were treated with 5 μg rICAM-1, rICAM-1-Ig or ovalbumin (OVA) (grade VI, Sigma, Deisenhofen, Germany) in phosphate buffer for 2 days before and 1, 2, 3, 4, 6 and 8 days after treatment with cyclophosphamide. Groups of 5 animals were killed before and 10 days after injection of cyclophosphamide. Total RNA was isolated from fresh pancreatic tissue by acid guanidinium thiocyanate-phenol-chloroform extraction and detection of mRNA was done by reverse transcriptase-PCR (RT-PCR) as described previously [16]. Specific primers for interferon (IFN)-gamma, interleukin (IL)-4 and β-actin were used (Clontech, Laboratories Inc., Palo Alto, Calif., USA). Control experiments verified that the chosen PCR cycle numbers were in the linear range of DNA amplification. PCR products were subjected to 2% agarose gel followed by hybridization with specific ^{32}P labelled probes. Measuring of signals was done with a phosphorimager (Fujix BAF 1000; Raytest, Straubenhardt, Germany) and results were expressed in relation to the β-actin signal, which was assigned a value of one.

Histological examination of pancreatic islets was done as described previously [17]. A total of 60 islets per group were analysed, at least 10 islets per animal. Only islets with a diameter of more than 100 μm were determined. Grading of islets was done as described [18].The histology score gives the mean infiltration grade of islets analysed.

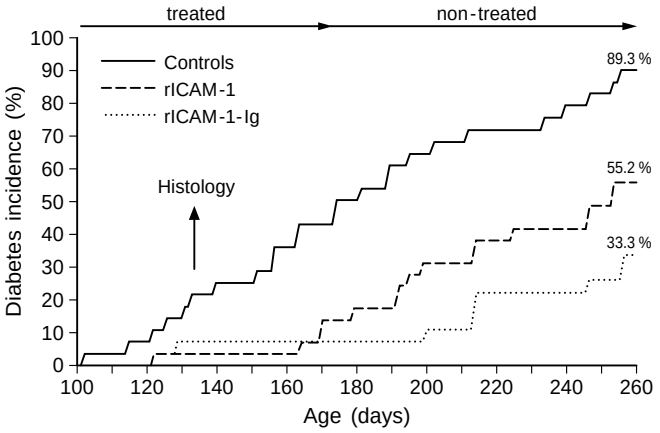

Fig. 1. Diabetes development during therapy with rICAM-1 preparations. NOD-mice were treated with i. p. injections of 15 μg rICAM-1 or rICAM-1-Ig per week beginning at day 30 until day 170. Control animals received i. p. injection of protein buffer

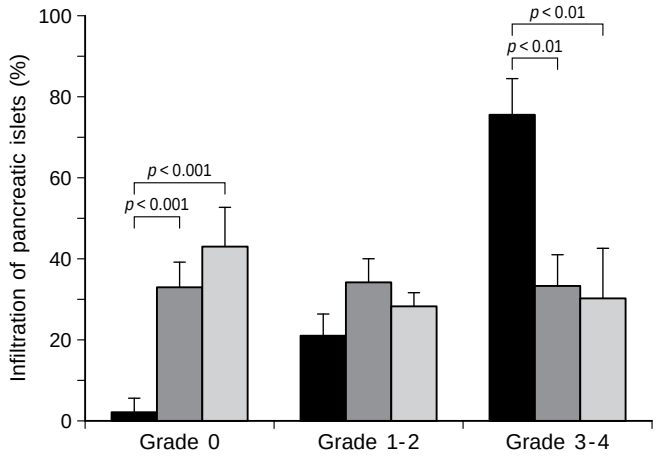

Fig. 2. Histological analysis of pancreatic islets. At day 132 infiltration of pancreatic islets were graded: Grade 0 was defined as no infiltration, grade 2–3 as moderate periinsulitis and grade 3–4 as severe intraislet infiltration. Mean values (+ SD) are shown. ■ controls; ▨ ICAM-1; □ rICAM-1-Ig

Statistical analysis. Differences in the incidence of diabetes and infiltration of pancreatic islets were analysed with the Mann-Whitney U test for non-parametric unpaired observations. The Log-Rank test was used for the analyses of time-dependent incidence of diabetes. Mean radioactive signals of RT-PCR products and ICAM-1-immunoreactivities in serum were compared by Wilcoxon U-test or Student's *t*-test, respectively.

Results

Treatment of NOD mice with recombinant solube ICAM-1. Recombinant ICAM-1 proteins with murine amino acid sequences were constructed and produced with comparable methods as we have used for human rICAM-1 and rICAM-1-Ig proteins [13]. Three different groups of NOD mice were treated with rICAM-1, rICAM-1-Ig or protein buffer as control. The first cases of overt diabetes were seen in the control group at day 100 and disease rate increased continously up to 89 % at day 260 (Fig. 1). In contrast, only one case of diabetes occurred in the rICAM-1 and two cases in the rICAM-1-Ig treated group during therapy (until day 170). Thereafter diabetes cases were also detected in the rICAM-1 treatment group, reaching a diabetes incidence of 55 % at the end of the experiment at day 260 ($p < 0.001$ compared with vehicle control group). Animals treated with rICAM-1-Ig showed further delay of development of diabetes and reached a rate of 33 % at 260 days ($p < 0.001$ or $p < 0.05$ compared with vehicle or rICAM-1 treated groups, respectively). During therapy no adverse effects or infections were noted, body weight development, leucocyte numbers or subpopulations did not differ between the three treatment groups.

These results demonstrate that giving soluble ICAM-1 can delay and suppress the development of autoimmune diabetes.

Histological analysis of pancreatic islets. Pancreatic islets of vehicle treated NOD mice showed severe infiltration (grade 3 and 4) in 76 % of cases (Fig. 2). Treatment with rICAM-1 or rICAM-1-Ig reduced significantly severe infiltration (33 % or 30 %, respectively) in comparison with control animals ($p < 0.01$)(Fig. 2). In addition, the percentage of non-infiltrated islets was higher in the treatment groups (33 % and 43 %) when compared with the vehicle control group (2 %, $p < 0.001$). These data indicate that treatment with soluble adhesion molecules prevents diabetes development by preventing infiltration of pancreatic islets.

Effect of soluble ICAM-1 on cytokine expression. To analyse the effect of soluble ICAM-1 treatment on cytokine gene expression during islet inflammation, the development of insulitis in NOD mice was synchronized by a single injection of cyclophosphamide. As reported before [16], there was an increase ($p < 0.01$) of IFN-gamma mRNA 10 days after cyclophosphamide injection in mice receiving treatment with a control protein (Fig. 3 a), and low concentrations of IL-4 mRNA expression persisted (Fig. 3 b). The ratio of T-helper type (Th1/Th2) cytokines showed a progression from an initial Th2- to a Th1-like cytokine pattern (Fig. 3 c). This rise in IFN-gamma gene expression has been previously correlated with the onset of beta-cell destructive cellular autoimmune reactivity in islets [16]. In comparison with the control group, which was treated with an indifferent protein, rICAM-1 increased ($p < 0.01$) the amount of IL-4 message formed and suppressed completely IFN-gamma expression in three out of five animals. Treatment with rICAM-1-

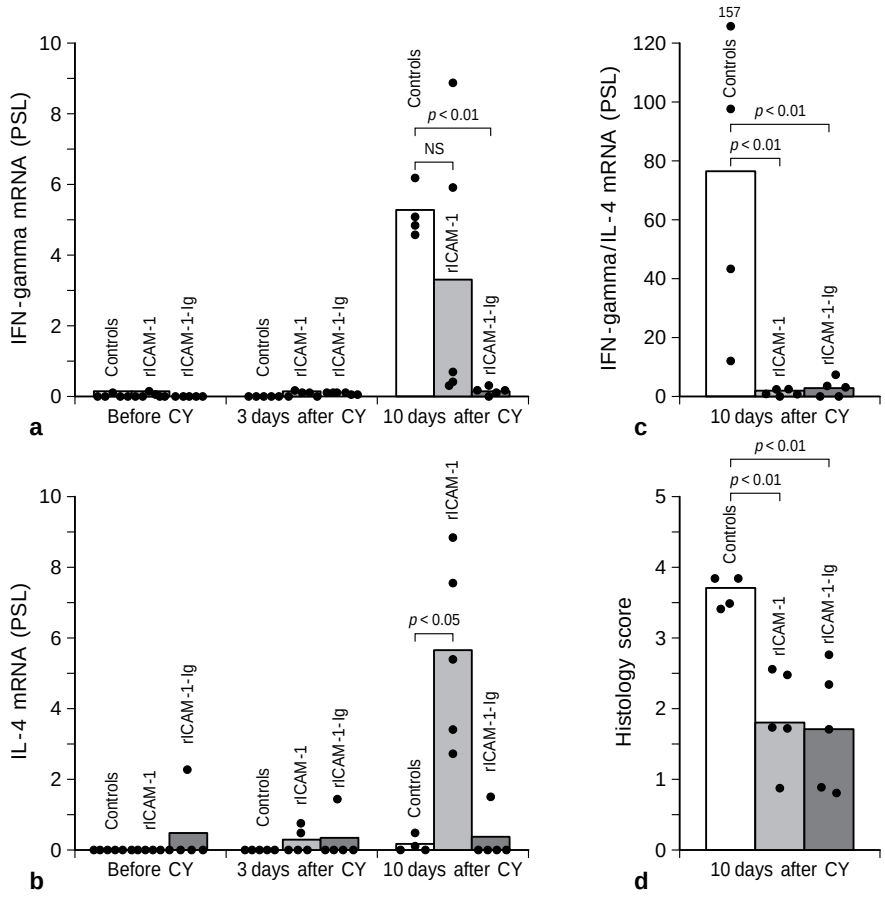

Fig. 3 a–d. Th1/Th2 cytokine expression and histology score. IFN-gamma (**a**) and IL-4 (**b**) cytokine gene expression was analysed in pancreas by RT-PCR before and 10 days after diabetes synchronization by injection of cyclophosphamide. The ratio of IFN-gamma/IL-4 mRNA (**c**) 10 days after cyclophoshamide injection was compared with the grade of the infiltration (**d**) in the same pancreatic tissues. Individual values (●) and mean values (bars) are shown. CY, cyclophosphamide

Ig suppressed IFN-gamma in all animals but did not interfere with IL-4 mRNA expression (Fig. 3). The ratio of IFN-gamma compared with IL-4 mRNA concentrations was calculated and is shown in Figure 3 c. Treatment with both recombinant ICAM-1 preparations prevented the shift toward Th1 cytokine gene expression in response to cyclophosphamide treatment (Fig. 3 c, $p < 0.01$). In parallel, histological examinations showed decreased ($p < 0.01$) insulitis scores in ICAM-1 and ICAM-1-Ig treated animals (Fig. 3 d). The individual Th1/Th2 cytokine ratio correlated ($p = 0.02$) with the corresponding islet histology score.

Serum concentrations of soluble ICAM-1. Finally, we determined whether treatment with soluble ICAM-1 would affect serum concentrations of soluble ICAM-1. After i. p. injection of 7.5 μg rICAM serum concentrations of soluble ICAM-1 showed a marked increase after 15 min ($p < 0.01$). This rise was transient and basal serum concentrations of soluble ICAM-1 were reached after 30 min. Serum concentrations of immunoreactive ICAM-1 showed an increase after prolonged treatment with rICAM-1 ($p < 0.01$ at 140 days therapy), data not shown. At 3 months after discontinuation of rICAM-1 injection ICAM-1-immunoreactivity was still ($p < 0.01$) elevated. In contrast, rICAM-1-Ig treated mice did not respond with an increase of circulating ICAM-1 (data not shown).

Discussion

The data reported here show for the first time that a "natural", spontaneously occurring autoimmune disease can be suppressed by injection of recombinant forms of syngeneic soluble adhesion molecules. NOD mice, which serve as an animal model for human Type I diabetes, were treated beginning at 30 days, when humoral autoimmunity is already present and first inflammatory changes are noted in panceratic islets [19]. Throughout the treatment development of diabetes was nearly completely suppressed, with concomitant inhibition of islet infiltration by mononuclear cells. A simple conclusion would be that soluble ICAM-1 interferes with leucocyte extravasation. Indeed, during the emigration of leucocytes the interac-

tion of ICAM-1 and β2-integrins (LFA-1, Mac-1) has an important role [6]. Also, hyperexpression of LFA-1 and ICAM-1 in infiltrated islets of NOD mice have been reported [17, 20–22] and the blockade of ICAM-1 and LFA-1 by injection of monoclonal antibodies to young NOD mice reduced the incidence of Type I and severity of infiltration [8].

Several observations argue against the hypothesis that soluble ICAM-1 directly interferes with lymphocyte adhesion to the endothelium. Recently, it has been reported that rICAM-1 inhibited cell-cell adhesion with an IC_{50} equally to 20–40 μmol/l, and the binding of ^{125}I-LFA-1 containing micelles to immobilised ICAM was blocked with an IC_{50} of approximately 200 nmol/l [23, 24]. These effective inhibitory concentrations are much higher than the concentration of circulating ICAM-1 (4 nmol/l) found in human serum. In previous studies we found that concentrations of 2–10 nmol/l rICAM-1 were sufficient to inhibit antigen- and autoantigen-induced T-cell proliferation in peripheral blood mononuclear cell from recent onset Type I diabetic patients [4]. These concentrations are clearly lower than required for inhibition of adhesion. In NOD mice the injection of rICAM-1 led to raised serum concentrations of 10–12 nmol/l, which were transient and lasted less than 30 min. These findings argue against the occurrence of a prolonged blockade of ICAM-1/LFA-1 interactions in mice receiving soluble rICAM-1. Therefore, the decreased number of mononuclear cells in islets of mice receiving rICAM-1 is probably not due to steric hindrance of adhesion processes during extravasation. It is more likely, the interaction of rICAM-1 with its receptor causes a functionally altered state, affecting autoimmune T-cell activation. This view is associated with our observation that treatment with rICAM-1 had immunomodulatory consequences, with inhibition of Th1 but no Th2 responses in the NOD mouse pancreas.

Injection of recombinant proteins could have induced antibody production against ICAM-1 which could be responsible for the protective effects. After termination of injection of recombinant proteins, however, an increase in the occurrence of diabetes was observed. Treatment of NOD mice with antibodies against ICAM-1 led to a permanent inhibition of the development of diabetes [25].

Studies of cytokine gene expression were carried out after cyclophosphamide treatment since this leads to synchronisation of islet inflammation and enables changes in cytokine patterns to be registered more easily [16]. The cyclophosphamide induced progression of peri-insulitis to destructive intra-insulitis occurs within 10 days and is associated with a shift from preferential IL-4 to strong IFN-gamma expression in islets [16]. This shift from Th2 to Th1 insulitis as determined by immunohistochemical analysis of cytokines in islets is paralled by a considerable increase of IFN-gamma mRNA in total pancreas or islet RNA, as shown previously [16, 26]. Both recombinant proteins inhibited insulitis and development of diabetes, however, rICAM-1 increased IL-4 concentrations and rICAM-1-Ig treatment reduced IFN-gamma concentrations. This illustrates the importance of the ratio of Th1 to Th2 cytokines as has been shown for recent onset Type I diabetic patients [27].

The relevance of the Th1 to Th2 cytokine mRNA ratio in the pancreas was underscored by the finding of a noticeable correlation with the individual islet histology score. A close correlation of the numerical ratio of IFN-gamma and IL-4 mRNA levels in total pancreas and insulitis grade has also been reported, for both BB rats and NOD mice [28, 29].

Another argument for an active immunomodulatory function of rICAM-1 comes from analysis of serum concentrations of endogenous soluble ICAM-1, after therapy. Surprisingly, prolonged treatment with rICAM-1 led to an increase of circulating ICAM-1 which persisted for at least 100 days after discontinuation of therapy. This upregulation of endogenous soluble ICAM-1 was observed in mice receiving rICAM-1 but not in the group receiving rICAM-1-Ig. Since rICAM-1and rICAM-1-Ig differ in the numbers of Ig-like domains, active regulation of serum ICAM-1 by exogenous recombinant ICAM-1 might be dependent on all five extracellular domains.

Multimerisation of ICAM-1 by fusing different extracellular domains on constant domains of immunoglobulins have been shown to be more efficient than monomeric rICAM-1 in inhibiting rhinovirus binding and infectivity [13,30]. Furthermore, multimeric ICAM-1 was approximately 1000 times more active in inhibiting autoantigen-specific T-cell proliferation [4]. Animals treated with ICAM-1-Ig were protected for at least 1 month beyond the cessation of therapy, and thereafter only few diabetic mice were observed. This protective effect is possibly due to the above mentioned funtional differences or a longer half-life of the proteins. Multimerisation of different adhesion molecules by fusing with IgG has shown therapeutical effects: Selectin-Ig chimera were effective in protection of neutrophil-mediated lung injury [31]. A fusion protein of VCAM-1 and IgG1 has been shown to interfere with the adoptive transfer of autoimmune diabetes by spleen cells [32]. These fusion proteins, however, were used only during a short time. In this study we showed a therapeutic influence of ICAM-1-Ig proteins over a period of 20 weeks without adverse effects.

In conclusion, this report describes for the first time the immunotherapeutical potential of soluble ICAM-1 proteins in vivo. At present, the exact mechanisms and potential benefit of this new approach are not known. During therapy no adverse effects were noted. Animals did not show any changes in leucocyte counts or subtypes, development of body weight was not affected and no infections were noted. Multi-

meric forms of these proteins might be superior since lower concentrations probably are necessary. This new type of immune intervention probably mimicks a natural feedback mechanism for limiting T-cell activation, in particular during Th1 responses through circulating soluble ICAM-1.

Acknowledgements. This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Ma1260/4) (S. Martin), the "Bennigsen-Foerder" grant from the Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, Düsseldorf (S. Martin), a grant from the Deutsche Diabetes-Gesellschaft (S. Martin) and institutional funds provided by the Bundesminister für Gesundheit, Bonn, and the Minister für Wissenschaft und Forschung des Landes Nordrhein-Westfalen, Düsseldorf.

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