Mucosal Addressin Is Required for the Development of Diabetes in Nonobese Diabetic Mice

Arno Hänninen, Ilkka Jaakkola and Sirpa Jalkanen

*J Immunol* 1998; 160:6018-6025; http://www.jimmunol.org/content/160/12/6018

**References**

This article cites 25 articles, 10 of which you can access for free at: http://www.jimmunol.org/content/160/12/6018.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Mucosal Addressin Is Required for the Development of Diabetes in Nonobese Diabetic Mice

Arno Hänninen, Ilkka Jaakkola, and Sirpa Jalkanen

Immune responses are best initiated in the environment of lymphoid tissues wherein circulating lymphocytes enter by interacting with endothelial adhesion molecules. In type 1 diabetes, immune responses against pancreatic islets develop, but the environment in which this occurs remains unidentified. To determine whether lymphocyte homing to lymphoid organs is involved in the pathogenesis of diabetes in nonobese diabetic (NOD) mice, we blocked the function of the mucosal addressin cell adhesion molecule-1 (MAdCAM-1), which is a vascular addressin-mediating lymphocyte homing into mucosal lymphoid tissues, in these mice. While ineffective if started later, a blockade started at 3 wk of age reduced the incidence of diabetes from 50% to 9% \((p < 0.01)\). This finding is associated with Peyer’s patch atrophy, a marked decrease of naive \((CD44^{low}CD45RB^{high})\) T lymphocytes, and a reduction in the relative numbers of memory \((CD44^{high})\) T lymphocytes in the spleen. The potential of these spleen cells to cause diabetes was diminished. Anti-MAdCAM-1 treatment also inhibited both lymphocyte entry into the pancreas and diabetes development in NOD/SCID recipients after the transfer of lymphocytes derived from the mesenteric lymph nodes of young, but not of diabetic, NOD donors. Therefore, MAdCAM-1 may be required during two distinct steps in an early phase of diabetes development: for the entry of naive lymphocytes into the lymphoid tissues in which diabetes-causing lymphocytes are originally primed, and for the subsequent homing of these lymphocytes into the pancreas. The role of MAdCAM-1 as a mucosal vascular addressin suggests that mucosal lymphoid tissues are involved in the initiation of pathologic immune responses in NOD mice. The Journal of Immunology, 1998, 160: 6018 – 6025.

The nonobese diabetic (NOD) mouse spontaneously develops an autoimmune syndrome similar to human insulin-dependent diabetes mellitus (IDDM) \((1,2)\). In IDDM, the development of autoimmunity against B cells is believed to require the predisposition of both genetic and environmental factors \((3)\). The activation of lymphocytes recognizing B cell determinants may therefore be triggered by environmental Ags, possibly via molecular mimicry \((4)\). Many environmental Ags pass via the gastrointestinal tract and are presented to lymphocytes in gut-associated lymphoid tissue (GALT). Lymphocytes seed to GALT and other lymph nodes during circulation by interacting with the surface molecules expressed on the postcapillary venules of these tissues. Some of these surface molecules exhibit tissue selectivity and, consequently, may selectively mediate lymphocyte homing to GALT or peripheral lymph nodes (PLNs) \((5)\). Mucosal addressin cell adhesion molecule-1 (MAdCAM-1), an endothelial glycoprotein whose extracellular portion consists of three distinct Ig domains and a mucin-like domain \((6)\), selectively mediates lymphocyte homing to GALT \((7)\). The activation of an immune response in GALT may require that lymphocytes have ready access to GALT during circulation, which would in turn be dependent upon the function of MAdCAM-1. Blocking its function may therefore offer a means to inhibit gut-associated immune responses and to test the impact of lymphocyte homing to GALT in the development of subsequent immune pathology such as that leading to spontaneous IDDM in the NOD mouse.

We and others have shown that the mucosal vascular addressin MAdCAM-1 is also constitutively expressed at low levels on pancreatic vasculature and, in conjunction with the appearance of lymphocyte infiltrates (insulitis) in pancreatic islets, becomes strongly induced on islet vessels \((8–10)\). MAdCAM-1 is one of the two principal vascular ligands of \(\alpha_4\) integrins \((11)\) and could, in theory, favor the homing of mucosal effector lymphocytes \((\alpha_4\beta_7^{high})\) and \(\alpha_4\beta_7^{low}\) to islets. Such lymphocytes predominate in islet infiltrates at early stages of the disease process \((12)\). Functionally, \(\alpha_4\) integrins are important in the development of diabetes both after adoptive cell transfer and spontaneously in the NOD mouse \((13–15)\). However, \(\alpha_4\) integrins interact not only with MAdCAM-1 but also with both the inducible endothelial adhesion molecule VCAM-1, which is expressed on the endothelium and on APCs such as dendritic cells \((16)\), and the extracellular matrix molecule fibronectin \((17)\). Therefore, \(\alpha_4\) integrins are involved not only in lymphocyte homing but also in lymphocyte interaction with APCs and, consequently, in T cell activation and in lymphocyte interaction with the extracellular matrix, making the interpretation of the mechanism underlying the observed effect more complicated. In contrast, the expression of MAdCAM-1 is more restricted \((10)\) to the venules of GALT, and MAdCAM-1 primarily serves as the vascular addressin directing lymphocyte entry into GALT \((7,11)\). Therefore, the MAdCAM-1 blockade may have a more restricted effect, primarily on lymphocyte homing to tissues in which it is expressed on the vascular endothelium.

To investigate the requirements of MAdCAM-1 for the entry of diabetogenic lymphocytes into the pancreas and for any earlier steps that were possibly involved in diabetes development, we...
initially blocked the function of MAdCAM-1 using Ab treatment in NOD/SCID mice receiving adoptive cell transfer of diabetogenic lymphocytes. We also treated spontaneously diabetic NOD mice in a similar manner from an early age onwards. The unexpected finding that MAdCAM-1 is not necessary for the development of diabetes after the adoptive transfer of diabetogenic spleen cells but is involved in diabetes development in the spontaneously diabetic NOD mice suggested that MAdCAM-1 may be important in the early steps of diabetes development. Our results imply that MAdCAM-1 may be involved in a step preceding the development of early diabetogenic lymphocytes in NOD mice and may also be involved in the entry of such lymphocytes into the pancreas.

Materials and Methods

Experimental animals

Our colony of NOD mice, which was bred and maintained in the animal facilities of the University of Turku (Turku, Finland) under specific pathogen-free conditions, originated from mice purchased from Bomholtgard (Ry, Denmark). In this colony, the cumulative incidence of diabetes by 180 days of age was 66% in females and 40% in males. Our colony of NOD/SCID mice was similarly maintained and originated from NOD/SCID mice that were kindly provided by Dr. M. Atkinson (University of Florida, Gainesville, FL).

Monoclonal Abs

We used the function-blocking mAbs MECA-367 (against MAdCAM-1) and Fib504 (against β3 integrins) provided by Dr. E.C. Butcher (Stanford University, Stanford, CA) to block the function of the mucosal vascular addressin MAdCAM-1 and the mucosal homing receptor α4β7 integrin in vivo. We used mAb 281.2 against mouse syndecan-1 (in adoptive cell transfer experiments with mesenteric lymph node (MLN) and spleen cells from diabetic donors, Fig. 1A) and mAb 9B5 against human CD44 (in all other experiments, Fig. 1, B–D and Fig. 2C) as species- and isotype-matched mAbs. We used the following mAbs to phenotype spleen and Peyer’s patch (PP) T cells: CRL-1911 for α4 integrins, MEL-14 for L-selectin, TIB-217 for CD11a, TIB-241 for CD44, and TIB-222 for CD25a (American Type Culture Collection, Manassas, VA) (all grown as hybridomas supernatants and concentrated by ammonium sulfate precipitation); Fib504 (see above) for β3 integrins, and mAb 16A for CD45RB (PharMingen, San Diego, CA).

Disease induction by adoptive cell transfer

Cells for adoptive cell transfer were isolated from aseptically removed spleens and MLNs representing GALT by squeezing the organs in glass homogenizers. Red cells were lysed by resuspending the spleen cells in 1 ml of sterile water for 10 s and then washed in 50 ml of RPMI 1640. After counting, 20 × 10^6 cells from pooled (4–8) spleens or MLNs derived from diabetic donors, Fig. 1A) and mAb 9B5 against human CD44 (in all other experiments, Fig. 1, B–D and Fig. 2C) as species- and isotype-matched mAbs. We used the following mAbs to phenotype spleen and Peyer’s patch (PP) T cells: CRL-1911 for α4 integrins, MEL-14 for L-selectin, TIB-217 for CD11a, TIB-241 for CD44, and TIB-222 for CD25a (American Type Culture Collection, Manassas, VA) (all grown as hybridoma supernatants and concentrated by ammonium sulfate precipitation); Fib504 (see above) for β3 integrins, and mAb 16A for CD45RB (PharMingen, San Diego, CA) was considered diabetic.

Treatment of mice with mAbs

Female NOD and NOD/SCID mice were treated with mAbs to investigate how MAdCAM-1 and α4β7 integrins affect the homing of diabetes-causing lymphocytes into the pancreas as well as possible earlier steps in the disease process. In adoptive cell transfer experiments, the recipients simultaneously received two i.p. injections of indicated mAbs (100 μg starting 2 h before the cell transfer) per wk. When cells from diabetic animals were used, the treatment was administered until diabetes occurred; when cells from young (6-wk-old) donors were used, the treatment was administered for 4 wk following the cell transfer. In experiments investigating the role of MAdCAM-1 and α4β7 integrins in the development of spontaneous diabetes and diabetes-causing lymphocytes, female NOD littermate mice received the indicated mAbs (in 100-μg aliquots) twice per wk starting at 3 wk of age (or, alternatively at 10 wk of age). Treatment was continued until 7 mo of age or until diabetes occurred.

For all experiments, mAbs were grown as serum-free supernatants of the corresponding hybridomas. Abs were concentrated from supernatants by salt precipitation (2.6 M NH₄SO₄), dialyzed against PBS, affinity purified in protein G columns (Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions, dialyzed, concentrated by lyophilization, and adjusted to a final concentration of 1 mg/ml.

Phenotyping of spleen and PP cells from mice treated with mAbs

To investigate how the MAdCAM-1 and α4β7 integrin blockade affect the phenotype of T cells during longstanding mAb treatment, female NOD mice that received mAbs starting at 3 or 10 wk of age and remained diabetes-free were killed at 7 mo of age. Spleen and PP cell suspensions were prepared from the pooled spleens or PPs of each group and were stained for flow cytometry using a FACScan (Becton Dickinson, San Jose, CA). The stainings were performed essentially as described previously (18) using antibodies (N-hydroxysuccinimide biotin; Calbiochem, La Jolla, CA) mAbs against indicated cell surface markers. Briefly, the cells were incubated with the biotinylated mAbs and washed. They were subsequently incubated with streptavidin-conjugated phycoerythrin (Becton Dickinson) as a second-step reagent that was used in combination with directly FITC-conjugated TIB-207 (anti-CD4) or Lyt2.2 (anti-CD8, PharMingen), washed, and fixed in paraformaldehyde. The staining of cells gave similar results in three different long-term treatment experiments in which the cells of three to five mice per treatment group were pooled each time before phenotyping.

Histochemistry and grading of islet infiltrates

For the morphologic analysis of PPs and other lymphoid organs, NOD mice that received longstanding mAb treatment (from 3 or 10 wk of age) and remained nondiabetic were killed at 7 mo of age; their PPs (and other lymphoid organs) were pooled within treatment groups (three or more mice per group in each experiment), treated for routine histology, and stained with hematoxylin and eosin.

To study the effect of mAb treatment on the development of lymphocytic infiltrates in pancreatic islets, NOD mice were treated from 2 wk of age (all females) or from 1 wk of age without knowledge of the sex (MEEA-367 group: four males plus one female; 9B5 group: three males plus two females) with anti-MAdCAM-1 or a control mAb (as explained above) until 7 wk of age. The mice were killed after 5 wk (at 12 wk of age). Islets in routinely processed and hematoxylin and eosin-stained sections of each pancreas were graded accordingly: 0 = no insulitis, 1 = peri-islet insulitis, 2 = insulitis occupying ≤50% of the islet area, 3 = insulitis occupying >50% of the islet area. Mean insulitis values represent the frequency of islets in these categories. A total of 35 to 60 islets were analyzed from each pancreas. The results from the two experiments were similar and were pooled.

Labeling of cells before in vivo transfer

For the early detection of lymphocytes that have homed into the pancreas of NOD/SCID recipients after the transfer of GALT cells from young (6-wk-old) donors, we labeled the cells before transfer with the fluorescent dye Dil (Molecular Probes, Leiden, the Netherlands). Briefly, Dil (1 mM in DMSO) was added in a 1:200 dilution to cells that had been adjusted to 10 million/ml in RPMI 1640. The cells were incubated at 37°C for 10 min, washed in RPMI 1640, and adjusted to 100 million/ml before transfer into NOD/SCID recipients. Starting at 2 h before cell transfer, recipients were simultaneously treated with the mAbs MECA-367 against MAdCAM-1 or 9B5 (control mAb) to investigate the involvement of MAdCAM-1 in the homing of GALT lymphocytes from young donors into the pancreas. Mice were killed at various timepoints after cell transfer (1, 3, 5, 7, and 14 days), and cryostat sections from each pancreas (three per group) were viewed under fluorescence microscopy (Olympus, Tokyo, Japan).

Results

Homing of early diabetogenic cells to the pancreas involves MAdCAM-1 interactions

To investigate the role of MAdCAM-1 in the homing of diabetogenic lymphocytes into the pancreas, we transferred spleen and MLN cells from diabetic NOD mice to female NOD/SCID mice receiving simultaneous Ab treatment. Treatment with the anti-MAdCAM-1 Ab MECA-367 did not affect the development of diabetes (Fig. 1A) or insulin (data not shown) in the recipients, indicating that the MAdCAM-1 blockade did not
prevent the homing of transferred cells to the pancreas. However, since the transferred cells were from overtly diabetic donors, many of the transferred effector cells were obviously already primed against islet Ags due to the established disease. Therefore, we also transferred MLN lymphocytes from young donors to NOD/SCID mice receiving Ab treatment to study the

FIGURE 1. MAdCAM-1 is required for pancreatic homing of diabetogenic GALT cells from young donors. A, Mice receiving spleen-representing (solid lines) or GALT-representing (broken lines) MLN lymphocytes from diabetic donors developed diabetes equally when treated with a control mAb (○), a mAb against β integrins (Fib504; ▲), a mAb against MAdCAM-1 (MECA-367; □), or both Fib504 and MECA-367 (▲□). B, Mice receiving MLN cells from young (6-wk-old) donors and anti-MAdCAM-1 mAb (□) developed diabetes less frequently than mice receiving the same cells and a control mAb (○). The data are pooled from three and two experiments, respectively, and the total number of mice in each treatment group is indicated. C, Level of lymphocyte infiltration in pancreatic islets (mean insulitis score) in mice that were treated with anti-MAdCAM-1 mAb (n = 5288 islets; gray bars) or a control mAb (n = 4206 islets; black bars) and subsequently killed at 4 wk after the transfer of MLN lymphocytes from young donors. D, Homing of the MLN lymphocytes (red fluorescence) of young donors to the pancreas during (left) or without (right) MAdCAM-1 blockade (original magnification = ×200).
potential requirement of MAdCAM-1 in the pancreatic homing of mucosal cells from an earlier phase of disease pathogenesis. In these transfers, anti-MAdCAM-1 treatment significantly reduced the incidence of diabetes, delayed the onset of diabetes \( (p < 0.02, \chi^2\text{ test}) \), and inhibited the formation of inflammatory infiltrates in islets (Fig. 1, B–C).

**FIGURE 2.** Longstanding anti-MAdCAM-1 treatment leads to PP hypoplasia and reduces the development of diabetes in NOD mice. A, PPs in the control-treated mice (left) have follicles with large germinal centers protruding above the surface of the bowel wall and are densely populated by lymphocytes. PPs in anti-MAdCAM-1-treated mice (right), although as numerous, are flat, have small follicles, and contain far fewer lymphocytes (original magnification = \( \times 100 \)). B, The subset distribution of T cells in the PPs of anti-MAdCAM-1-treated mice (contour histograms) is different from that seen in the control-treated mice (gray histograms). PPs are depleted almost completely of T cells expressing high levels of CD8 (arrow) and the percentage of naive (CD44\(^{lo}\)CD45RB\(^{lo}\)) CD4 T cells is markedly decreased, which leads to a relative increase in the number of memory (CD44\(^{hi}\)CD45RB\(^{lo}\)) CD4 T cells (arrows). The percentages of positive cells in anti-MAdCAM-1-treated mice (m) and control Ab-treated mice (co) are indicated. The percentages of CD44\(^{hi}\) and CD45RB\(^{lo}\) cells (indicated by dashed lines and arrows) are 34% and 65%, respectively, in anti-MAdCAM-1-treated mice and 2% and 37%, respectively, in control mAb-treated mice. C, The development of diabetes is significantly inhibited in mice receiving anti-MAdCAM-1 treatment (□) compared with mice receiving control mAb 9B5 (○), mAb Fib504 (▲), or PBS (●). The data are pooled from three long-term anti-MAdCAM-1 treatment experiments. The total number of mice in each treatment group is indicated.
Due to a relatively long time lag before the formation of easily detectable inflammatory infiltrates in these transfers, we also labeled MLN cells from young donors with the fluorescent dye DiI to trace the first cells which home into the pancreas after transfer. At 7 days after the transfer, fluorescent cells were frequently detected perivascularly and periductally in the pancreata of recipients treated with a control Ab, whereas only sporadic fluorescent cells were detected in the pancreata of recipients treated with anti-MAdCAM-1 Ab (Fig. 1D). This finding indicates that the homing of early developing diabetogenic cells into the pancreas depends, in part, upon MAdCAM-1.

MAdCAM-1 affects the size of GALT and is required for the development of diabetes

Under physiologic conditions, MAdCAM-1 is expressed preferentially on the high endothelial venules of organized mucosal lymphoid tissues and is required for the entry of lymphocytes into GALT (5, 7). To test how longstanding anti-MAdCAM-1 treatment affects GALT and the development of diabetogenic effector lymphocytes, we treated NOD mice continuously with i.p. injections of MECA-367 or a control mAb in two sets of experiments and followed the development of diabetes until 7 mo of age. In the first set of experiments, the treatment was started at 21 days of age, when there are no lymphocyte infiltrates in pancreatic islets. In the second set, the treatment was started at 10 wk of age with preexisting infiltrates in islets. The size and appearance of the spleen, MLNs, and PLNs were comparable in all animals. However, PPs were much smaller and contained much fewer lymphocytes in the group of animals that had received anti-MAdCAM-1 treatment from 3 wk of age compared with PPs in other groups of mice (Fig. 2A). Thus, the PPs in this group resembled PPs in mice that are genetically deficient for β7 integrins (19). In our mice, PPs lacked T cells expressing high levels of CD8, and the relative number of CD4 T cells of the memory cell phenotype (CD44highCD45RBlow) (Fig. 2B) was clearly increased. Anti-MAdCAM-1 treatment that was initiated at 3 wk of age also resulted in an almost complete reduction in the development of diabetes (p < 0.01 compared with the rat IgG2a control mAb 9B5) (Fig. 2C). A similar treatment started at 10 wk of age neither affected the size and cellular composition of PPs (data not shown) nor interfered with the development of diabetes. Treatment with a mAb against β7 integrins was ineffective at all ages. This lack of effectiveness may reflect the...
The fact that α4/β7 and the mAbs against it control lymphocyte homing to PPs less effectively than MAdCAM-1 and mAb MECA-367 (18, 20). Anti-MAdCAM-1 treatment also significantly reduced lymphocyte infiltration in the pancreatic islets (Fig. 3).

MAdCAM-1 blockade inhibits the development of diabetes-causing lymphocytes

The homing of early developing diabetogenic effector lymphocytes to the pancreas was partly dependent upon MAdCAM-1, which could explain why the anti-MAdCAM-1 treatment started at 3 wk of age inhibited the development of diabetes. To determine whether diabetogenic cells were simply kept outside the pancreas during the MAdCAM-1 blockade or if the development of diabetogenic effector lymphocytes was also compromised, we transferred spleen cells from those NOD female mice treated with mAbs to NOD/SCID recipients (21). The recipients of spleen cells derived from anti-MAdCAM-1-treated mice became diabetic with a significantly longer time lag (mean 6.2 wk vs 4.3 wk) than the recipients of spleen cells derived from the control mAb-treated mice (Fig. 4A; p < 0.01 for the difference in the proportion of diabetic mice at 4 wk posttransfer, $\chi^2$ test). In the spleen, the expression of CD44 and CD11a and the expression of α4 integrins was lower among the CD4 T cells of mice treated with anti-MAdCAM-1 mAb than among the CD4 T cells of control mice (Fig. 4B).

Discussion

In the present study, we describe the requirement of MAdCAM-1 in the recirculation and homing of lymphocytes before their accumulation in pancreatic islets. Unlike its lymphocyte ligands, the α4 integrins, which are involved in a variety of cell to cell interactions during immune responses (13, 14), the function of MAdCAM-1 is restricted to the homing of lymphocytes. This specificity may explain the differences in the effect of the MAdCAM-1 blockade when compared with the blockade of α4 integrins: while the MAdCAM-1 blockade was effective only if started before the onset of insulitis and not when delayed until insulitis was present or when
given after the adoptive transfer of cells from diabetic donors, the blockade of \( \alpha_4 \) integrins was effective against both the development of spontaneous diabetes in NOD mice and the transfer of diabetes by spleen cells from overtly diabetic mice (15–17). Therefore, MAdCAM-1 is exclusively involved in an early phase of diabetes pathogenesis in NOD mice.

As naive lymphocytes become activated in secondary lymphoid organs (such as GALT, PLNs, and the spleen) with the help of professional APCs that have captured and processed antigenic material, lymphocyte homing to secondary lymphoid organs is critical for the development of immune responses (22). A primary hypothesis behind our work was that we should be able to inhibit diabetes by inhibiting lymphocyte homing into those lymphoid organs in which the priming of putative diabetogenic lymphocytes occurs. Since environmental factors have been implicated in diabetes pathogenesis (1–4), we hypothesized that lymphocyte homing to, and, consequently, priming in the GALT may play a role in diabetes pathogenesis. The results of the long-term MAdCAM-1 blockade experiments supported the idea that GALT may initially be an important site for the priming of diabetogenic lymphocytes. Furthermore, the adoptive transfer of diabetes with spleen cells isolated from anti-MAdCAM-1-treated mice suggested that these spleen cells had not acquired a diabetogenic potential equal to that of spleen cells of normal NOD mice. Since MAdCAM-1 is not involved in lymphocyte entry into the spleen (23), this observation indicates that anti-MAdCAM-1 treatment probably interfered with a step in the development of diabetogenic lymphocytes that is proximal to their accumulation in the spleen. Such a step could involve a mechanism in which MAdCAM-1 is required, i.e., lymphocyte homing to lymph nodes, especially to those in the gut region. That MAdCAM-1 is essential for lymphocyte entry into PPs was evidenced by the fact that the MAdCAM-1 blockade reduced the size of PPs and the relative numbers of naive lymphocytes in PPs. The reduced traffic of naive lymphocytes via PPs may, in turn, have reduced the chances of naive lymphocytes to become primed and, consequently, to develop into activated and/or Ag-experienced lymphocytes. This possibility was supported by the finding that both the expression of the memory markers CD44 and CD11a and the expression of the finding that both the expression of the memory markers CD44 and CD11a and the expression of the finding that both the expression of the memory markers CD44 and CD11a and the expression of

References