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EC3, a Heterodimeric Disintegrin from *Echis carinatus,* Inhibits Human and Murine α4 Integrin and Attenuates Lymphocyte Infiltration of Langerhans Islets in Pancreas and Salivary Glands in Nonobese Diabetic Mice

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The venom of *Echis carinatus suchoreki* contains a monomeric disintegrin echistatin (Mr 5,500 Da) that strongly inhibits αIIbβ3, αvβ3, and α5β1 integrins and a heterodimeric disintegrin called EC3 (M_r 14,762 Da). At nanomolar concentration, EC3 inhibits adhesion of human cell lines expressing α4β1 and α4β7 to immobilized VCAM-1; it has a lower inhibitory effect on α5β1-mediated cell adhesion. In this study, we demonstrated that EC3, in contrast to echistatin, inhibited binding of monoclonal anti-α4 and anti-α5 antibodies to cells expressing α4β7. In a dose-dependent manner and to the same extent, EC3 inhibited adhesion of Jurkat cells and murine splenic lymphocytes to immobilized VCAM-1, whereas echistatin was not active. EC3 injected intraperitoneally into nonobese diabetic (NOD mice) suppressed development of insulitis and sialoadenitis, whereas echistatin had no significant effect. We propose that the effect of EC3 is mediated, at least, in part, by blocking α4β1 and α4β7 on murine lymphocytes.

Disintegrins are low molecular weight, cysteine-rich proteins isolated from the venoms of various vipers. They occur either in monomeric or dimeric forms. Monomeric disintegrins include trigramin, albolabrin, kistrin, flavoridin, echistatin, eristostatin, and bitistatin and are potent inhibitors of platelet aggregation (1). They all contain a RGD or KGD motif and are maintained in an appropriate conformation by intramolecular S-S bridges (2, 3). Echistatin isolated from *Echis carinatus suchoreki* venom (4) potently inhibits αIIbβ3, avβ3, and α5β1 integrin. The integrin recognition sites of echistatin are located in the hairpin loop and C-terminus (5).

More recently we isolated and characterized a novel heterodimeric disintegrin from *Echis carinatus suchoreki* venom (6). This disintegrin, named EC3, has a molecular weight of 14,762 Da and it is composed of two subunits, EC3A and EC3B, linked by intramolecular S–S bonds. Each subunit contains 67 residues, including 10 cysteines, and displays a high degree of homology between themselves and with other disintegrins. The hairpin loop of echistatin KRARGDDMDDY is substituted in EC3A and EC3B with KRAVGDDVDDY and KRAMLDGLNDY, respectively. EC3 inhibited the adhesion of cells expressing α4β1 and α4β7 to natural ligands VCAM-1, MadCAM with IC_{50} = 6–30 nM, and adhesion of K562 cells (α5β1) to fibronectin with IC_{50} = 150 nM. It did not inhibit adhesion of cells transfected with αvβ3 to vitronectin. Activity appeared to reside in subunit B containing MLDG sequence. Echistatin had no effect on α4β1 and α4β7 integrins (6).

Nonobese diabetic (NOD) mice spontaneously develop age-related lymphocyte infiltration of Langerhans islets and salivary glands which resembles pathological alteration in human pancreas in the course of diabetes type I and in salivary glands during the course of Sjögren syndrome. T-lymphocytes from these animals appear to be predominantly involved in infiltration. Splenic T-lymphocytes can transfer diabetes into nondiabetic recipient mice (7). Monoclonal antibodies to α4β1 (5) and to α4β7 integrin (9) inhibit disease progress in the pancreas but have no signifi-

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EC3A	NSVHPCCDPVTCEPREGEHCISGPCCRNCYFLRAGTVCKRA	*VGD*	DVDDYCSGITPDCPRNRYKGKED
EC3B	NSVHPCCDPVTCEPREGEHCISGPCCRNCKFLNAGTICKRA	*MLD*	GLNDYCTGKSSDCPRNRYKGKED
Echistatin	ECESGPCCRNCKFLKEGTICKRA	*RGD*	DMDDYCNGKTCDCPRNHKGPAT

FIG. 1. Amino acid sequences of monomeric disintegrin echistatin and of subunits A and B of heterodimeric EC3.

cant effect on the progression of the disease in salivary glands.

This study demonstrates that EC3 competes with monoclonal antibodies recognizing both $\alpha 4$ and $\beta 7$ integrins and that it blocks binding of Jurkat cells and murine splenic lymphocytes to immobilized VCAM1 with equal potency. Moreover, we found that EC3, in contrast to echistatin, attenuates lymphocyte infiltration of Langerhans islets and of salivary ducts in NOD mice. We propose that this effect of EC3 is mediated at least, in part, by the inhibition of integrins expressed on murine leukocytes.

MATERIALS AND METHODS

Reagents. Both disintegrins echistatin (10) and EC3 (6) have been purified from the venom of *Echis carinatus suchoreki* purchased from Latoxan, Valence, France (Fig. 1). The purity was confirmed by SDS–PAGE and mass spectrometry. Purified human and murine recombinant VCAM-1 was provided by Dr. Mark Renz, Genentech (San Francisco, CA) and by Dr. Roy R. Lobb, Biogen (Cambridge, MA). Monoclonal antibodies against murine von Willebrand Factor, CD31, VCAM-1, ICAM-1, ICAM2 and MadCAM-1 and monoclonal antibody against human $\beta 7$ and αL were purchased from Pharmingen (San Diego, CA). Monoclonal antibody against human $\beta 1$ integrin was from Immunotech Inc. (Westbrook, ME). Fluoresceine isothiocyanate-conjugated anti-mouse IgG for flow cytometry was purchased from Jackson Immune Research (West Grove, PA). All other reagents including ethyl alcohol, acetone, xilene, hematoxylin–eosin, LiCa3, OCT compound, cytoseal and glass slides were purchased from Fisher Scientific Co. (Pittsburgh, PA).

Cell lines. Jurkat cells expressing $\alpha 4\beta 1$ and $\alpha 5\beta 1$ and Ramos cells $\alpha 4\beta 1$ were purchased from ATCC (Manassas, VA). RPMI 8866 cells expressing $\alpha 4\beta 7$ were generously provided by Dr. Garcia-Padro, Madrid, Spain.

Total murine splenic lymphocytes. The spleens of 11-week-old mice were removed under sterile conditions by placing them in RPMI medium and gently shaking. After incubation for 15 minutes at 37°C in 5% CO_2, the cells were separated from the debris on Percoll gradient.

Flow cytometry. Samples for flow cytometry analysis were prepared as described (6) and analyzed in a Coulter Epics flow cytometer (Miami, FL).

Cell adhesion. Adhesion of human cell lines or total murine spleen lymphocytes, labeled with 5-chloromethylfluorescein diacetate was performed as described previously (6). Briefly, 1 μg of VCAM-1 or EC3 were plated into a 96-well plate. Then cells were added and incubated for 2 h with echistatin or EC3. The nonadhering cells were removed by washing and bound cells were lysed by the addition of 0.5% Triton X-100. In parallel, a standard curve was prepared in the same plate using known concentration of labeled cells. The plates were read using a Cytofluor 2350 fluorescence plate reader (Millipore, Bedford, MA) with a 485 nm excitation filter and a 530-nm emission filter.

Animal experiments. All experiments were performed with the approval IACUC committee in Temple University Medical School. Female NOD mice from 1 to 3 weeks of age were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were divided into three groups and were injected intraperitoneally with 0.5 ml PBS, 0.5 ml echistatin, or 0.5 ml EC3. EC3 and echistatin were given in a dose 3 μg and 1 μg, respectively. The EC3 and echistatin were equivalent at the molar level since the molecular mass of EC3 is approximately three times higher than that of echistatin. The treatment of animals lasted from 5 to 9 weeks.

After the treatment, the animals were sacrificed by exposure to CO_2. Some control animals were also sacrificed after 4 and 8 weeks. The pancreas and salivary glands were removed, placed in TO compound, frozen, and stored at −70°C. For histological analysis, 5 mm sections of frozen tissues were fixed with acetone. For detection of infiltrating lymphocytes, sections were stained with hematoxylin/eosine, cut by cryostat, air dried, and observed by light microscopy. Three groups of control, nontreated animals were killed after 4, 8, and 12 weeks of age to test for the expression of antigens in pancreatic and salivary tissue.

Statistical analysis. The degree of lymphocyte infiltration was measured in 27 islet samples from 8 control mice, 28 islet samples from 10 mice treated with EC3, and 29 islet samples from 10 mice treated with echistatin in each islet quantitated. The degree of infiltration was graded as 0, +, ++, +++. Similarly, the degree of lymphocyte infiltration was evaluated in salivary gland samples from 8 control mice, from 9 mice treated with EC3, and from 10 mice treated with echistatin. Each salivary gland sample was also graded by the degree of infiltration (+, ++, +++). In most experiments, two investigators observed tissue sections independently.

The degree of infiltration of islets and salivary glands was compared between the three groups. All data were transformed using ranks adjusted for ties; this was necessary due to the ordinal scales used for evaluation. Each analysis was carried out as a randomized block design with animals used as blocks (11). This procedure adjusts for multiple samples per animal. Differences were first assessed between treatment groups and then (for islet samples only) as a binary outcome (infiltration vs. non-infiltration). Multiple comparisons among means were carried out using a Bonferroni adjustment (11).

Due to the lack of independence among samples (i.e., multiple samples per animal), it was necessary to model the number of islet samples positive for infiltration using a Poisson distribution. A generalized linear model was used with a log link function and a Poisson distribution. This analysis makes no assumption regarding independent observations.

RESULTS AND DISCUSSION

Previous experiments (6) demonstrated that EC3 is a potent inhibitor of adhesion of Jurkat cells expressing $\alpha 4\beta 1$, and RPMI 8866 cells expressing $\alpha 4\beta 1$ to immobilized VCAM-1 and MadCAM, respectively. Figure 2 shows that EC3, in contrast to echistatin, inhibited binding of monoclonal antibodies directed against $\alpha 4$ and $\beta 7$ integrin to Ramos cells. Binding of antibodies against $\alpha 4$ was not affected by either disintegrin. Monoclonal antibodies against $\alpha 4$ and $\beta 7$ integrins inhibited adhesion of RPMI 8866 cells to immobilized

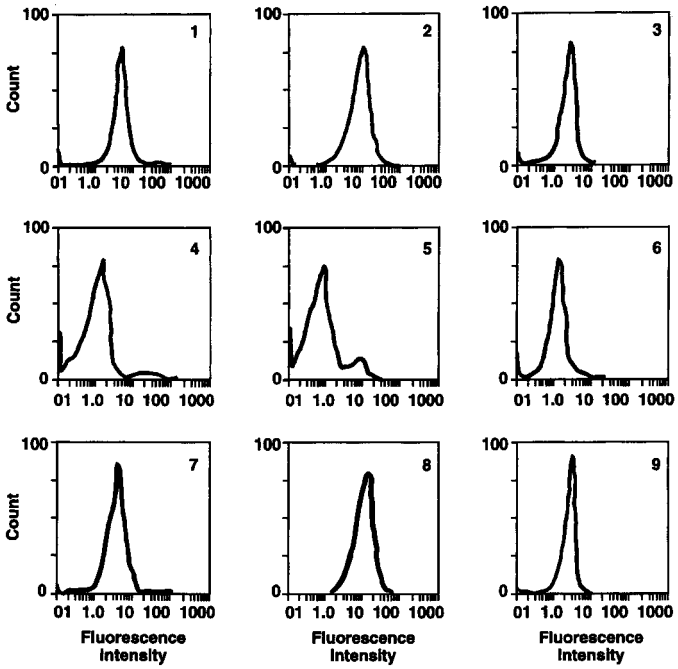

FIG. 2. Effect of disintegrins EC3 and echistatin on the binding of monoclonal antibodies against α4, β1, and β7 to human cell lines (Ramos and RPMI 8866 cells). 200 μl of cell suspensions were incubated (1×10^6 cells per ml) for 30 min at 4°C with BSA or disintegrin with 5 μg monoclonal antibodies (mab). After washing, the excess of disintegrin cells were resuspended in 200 μl HBBS and incubated. After 30 min incubation, cells were washed; FITC-labeled goat anti-mouse IgG antibody was added and analyzed by flow cytometry. Control Ramos cells + anti-β1 mab (stained cells, 96%). (2) Control RPMI 8866 cells, anti-α4 mab (stained cells, 83%). (3) Control RPMI 8866 cells + anti-β7 mab (stained cells, 90%). (4) Ramos cells + EC3, 10 μM + anti-β1 mab (stained cells, 45%). (5) RPMI 8866 cells + EC3 + anti-α4 mab (stained cells, 45%). (6) RPMI 8866 cells + EC3 10 μM + anti-β7 mab (stained cells, 35%). (7) Ramos cells + echistatin, 10 μM + anti-β1 mab (stained cells, 78%). (8) RPMI 8866 cells + echistatin 10 μM + anti-α4 mab (stained cells, 90%). (9) RPMI 8866 cells + echistatin + anti-β7 mab (stained cells, 67%). Binding of anti-αL antibody to Ramos and RPMI cells was not affected by EC3 and echistatin (not shown).

EC3 (data not shown). Figures 3 and 4 show that EC3 had similar inhibitory effect on the binding of the Jurkat cells and on the binding of the suspension of murine total spleen leukocytes to immobilized VCAM1. Echistatin was not active in either system.

In further experiments, we investigated the effect of EC3 and echistatin on the spontaneously developing lymphocyte infiltration of pancreatic Langerhans islets and salivary glands of NOD mice. In these experiments, mice were divided into 3 groups and each group was treated with PBS, echistatin, and/or EC3 for 9 weeks. Table 1 shows the effect of EC3 and echistatin on the infiltration of Langerhans islets in pancreas by lymphocytes. None of the EC3 treated mice had an infiltration score of 3+, whereas 9 of 25 control mice islets had an infiltration score of 3+. Conversely, 10 of 29 islets from NOD mice treated with EC3 showed no

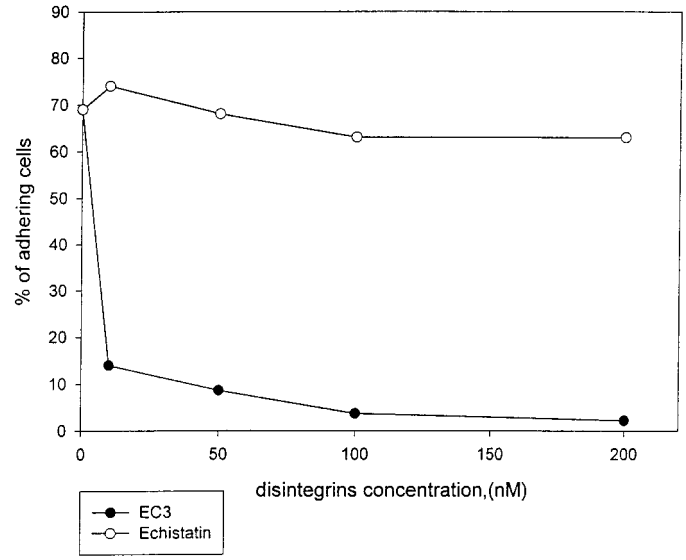

FIG. 3. Inhibitory effect of echistatin and EC3 on the adhesion of Jurkat cells to immobilized human VCAM-1. EC3, closed circles; echistatin, open circles. For other explanations, see Materials and Methods. Mean values of two replicate experiments are shown.

infiltration, whereas only one of 25 control mice islets showed no infiltration. The differences between the control and EC3 were highly significant. Echistatin tends to reduce small peri-insular infiltration without affecting the formation of larger infiltrates (Table 1). In order to evaluate infiltration of salivary glands, the number of inflammatory foci were counted (Table 2). The total number of infiltration foci decreased from 34 in control mice, to 25 in echistatin treated mice, and to

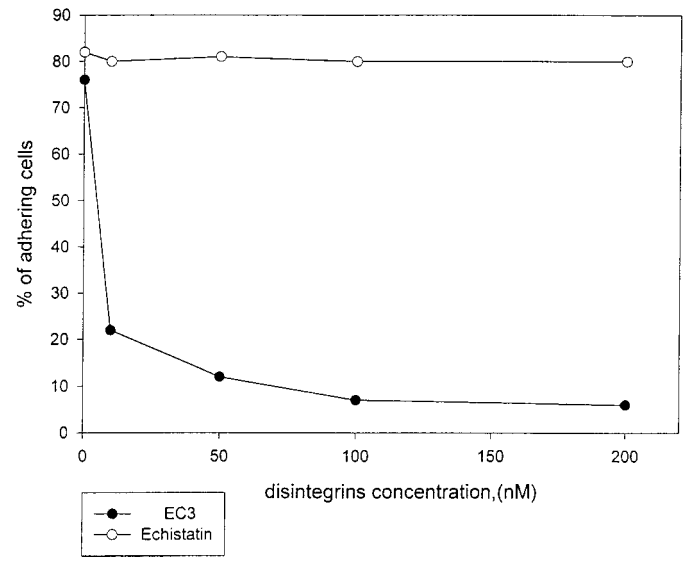

FIG. 4. Inhibitory effect of echistatin and EC3 on the adhesion of total murine splenic lymphocytes to immobilized murine recombinant VCAM-1. See Fig. 3 and Materials and Methods for other explanations.

14 in EC3 treated mice. The differences were highly significant statistically. Echistatin appeared to reduce small periductal infiltrates, but this effect was statistically insignificant. There were no significant relationships between the occurrence of infiltration and the treatment group in islet samples. In summary, EC3 inhibited formation of all type of infiltrates in both pancreas and in salivary glands, whereas echistatin was not effective.

Staining of tissue sections of control NOD mice by various antibodies demonstrated that endothelia of salivary glands and of Langerhans islets expressed von Willebrand Factor, CD31, ICAM1, and ICAM2 during 4, 8, and 12 weeks of age (data not shown). During progression of disease, MadCAM was expressed in endothelia of pancreatic islets but not in endothelia of salivary glands. VCAM-1 was expressed on endothelia of pancreatic islets only after 8 weeks of age and in salivary glands after 12 weeks (data not shown). This observation is consistent with the data reported by Michie *et al.* (12) except in the NOD mice tested in our laboratory where the infiltration of the salivary gland was observed earlier.

Yang *et al.* (1994) observed the inhibitory effect of anti-$\alpha 4$ antibodies on lymphocyte infiltration of Langerhans islets but not on the infiltration of salivary glands. In contrast, EC3 inhibited lymphocyte infiltration in both organs. This difference may be due to the fact that EC3, being a very potent inhibitor of $\alpha 4\beta 1$ and $\alpha 4\beta 7$ at 6–30 nM, also inhibits $\alpha 5\beta 1$ at 150 μM.

Most recently, Uniyal *et al.* (1999) reported that leukocytes utilize both $\alpha 4$ and $\alpha 5$ integrins for the infiltration of Langerhans islets in NOD mice. Therefore, disintegrins blocking both $\alpha 4\beta 1$, $\alpha 4\beta 7$, and $\alpha 5\beta 1$ integrins may have a stronger effect than monoclonal antibodies selectively blocking $\alpha 4\beta 1$ or $\alpha 4\beta 7$. However, the selective blocking of $\alpha 5\beta 1$ by echistatin is not sufficient to attenuate infiltration. It has been suggested that interaction of leukocytes expressing $\alpha L\beta 2$ integrin with endothelial ICAM-1 is relevant to the infiltration of salivary glands of NOD mice (14,15). However, our data indicate that EC3 is a weak inhibitor of the binding of $\beta 2$ expressing cells to ICAM ($IC_{50} > 1$ μl).

TABLE 1

Effect of Disintegrins on the Infiltration of Langerhans Islets in Pancreas of NOD Mice

Group	Number of islets	Infiltration score			
		−	+	++	+++
Control (n = 8)	25	1	5	10	9
EC3 (n = 10)	32	10	12	7	3
Echistatin (n = 10)	27	5	2	10	10

Note. In this experiment, animals received treatment for 9 weeks. Infiltration score: −, no infiltration; +, infiltration at the periphery of islets; ++, 1/3 of the islet infiltrated; +++, 1/2 (or more) islet infiltrated. Control vs EC3, $P = 0.0045$; control vs echistatin, $P = 1.0000$; EC3 vs echistatin, $P = 0.028$.

TABLE 2

Effect of Disintegrins on the Infiltration of Salivary Ducts by Lymphocytes

Group	Number of foci	Infiltration score			
		−	+	++	+++
Control (n = 8)	34	0	0	30	4
EC3 (n = 10)	14	0	10	4	0
Echistatin (n = 10)	25	0	7	15	3

Note. In this experiment, animals received treatment for 9 weeks. Infiltration score: −, no filtration; +, infiltration at the periphery of the duct; ++, 1/3 of the islet infiltrated; +++, 1/2 (or more) of the duct infiltrated. Control vs EC3, $P = 0.00001$; control vs echistatin, $P = 0.1594$; EC3 vs echistatin, $P = 0.0036$.

In summary, this data provides evidence that EC3 is an active molecule *in vivo* as well as *in vitro.* EC3 attenuates lymphocyte infiltration of pancreatic Langerhans islets and salivary glands in NOD mice. It appears that the EC3 effect is mediated at least in part by the inhibition of binding of $\alpha 4\beta 1$, $\alpha 4\beta 7$, and $\alpha 5\beta 1$ expressing T-lymphocytes to endothelial VCAM-1. In addition, our studies show that NOD mice represent a useful model to study the inhibitory effect of disintegrins and short peptides on the development of autoimmune disease.

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