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EndoS Reduces the Pathogenicity of Anti-mCOL7 IgG through Reduced Binding of Immune Complexes to Neutrophils

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Abstract

Endo-β-N-acetylglucosaminidase (EndoS) has been shown to act as a potent pathogen-derived immunomodulatory molecule in autoimmune diseases. Here we investigated how EndoS treatment reduces the pathogenicity of rabbit anti-mCOL7 IgG using different experimental models of epidermolysis bullosa acquisita (EBA). Our results show that the EndoS treatment does not interfere with the binding of the antibody to the antigen but reduces immune complex (IC)-mediated neutrophil activation by impairing the binding of the IC to FcγR on neutrophils. On the basis of this newly identified EndoS-mediated mechanism we hope to develop new strategies in the treatment of the disease.

Introduction

Endo-β-N-acetylglucosaminidase (EndoS) is an endoglycosidase secreted by Streptococcus pyogenes that specifically hydrolyzes the β-1,4-di-N-acetylchitobiose core of the asparagine-linked glycan of human IgG [1]. It has evolved as a powerful tool of Streptococcus pyogenes to combat human humoral defense system. EndoS has been shown to hydrolyze efficiently native IgG both in vitro and in vivo, which make it a good candidate for a pathogen-derived immunomodulatory molecule [2]. So far, the therapeutic efficacy has been shown in experimental models of many autoimmune diseases, including antibody-induced arthritis [3], immune-thrombocytopenic purpura [2], lupus-like disease [4], glomerulonephritis [5], and autoimmune hemolyis [6] This suggest that EndoS can be used as a powerful therapeutic molecule in the treatment of autoimmune diseases, especially those involving autoantibodies.

Recently, we have shown that treatment of EndoS results in the modulation of experimental epidermolysis bullosa acquisita (EBA) [7], an autoimmune skin blistering disease mediated by autoantibodies against type VII collagen [8]. The pathogenesis of this disorder involves the Fc portion of the autoantibodies as well as the complement system both mediating the activation of neutrophils which act as essential executor of tissue damage [9–11]. Several modeling systems of EBA have been established allowing the precise analysis of the disease during different phases. These models include the active immunization of mice with type VII collagen, a passive transfer of anti-mCOL7 IgG into animals, as well as an ex vivo model on cryosections and an in vitro neutrophil activation assay [10–13].

In this study, we applied the ex vivo model and the in vitro neutrophil activation system to analyze the cellular and molecular mechanisms by which the EndoS treatment reduced the pathogenicity of rabbit anti-mCOL7.

Materials and Methods

Rabbit Anti-mCOL7 IgG Preparation

Pathogenic rabbit anti-mCOL7 IgG was obtained from a commercial supplier (Eurogentec, Koln, Germany) and generated as previously described [10]. In brief, New Zealand white rabbits were immunized with recombinant forms of the glutathione S-transferase (GST)-tagged NC1 domain of mCOL7, and anti-mCOL7 IgG was purified from the rabbit serum affinity chromatography using protein G. Recombinantly expressed GST-EndoS was prepared as described [14]. The expression construct can for non for profit use be obtained at www.addgene.org/44655/. EndoS-treated rabbit anti-mCOL7 IgG was prepared as described previously [7]. Rabbit control IgG was prepared from healthy New Zealand white rabbit serum.
Evaluation of Dermal-epidermal Separation

Skin dermal-epidermal separation was evaluated using an modified ex vivo model [7]. Briefly, 6 μM cryosections prepared from C57BL/6j mouse tail skin were placed in the center of a Superfrost Plus microscope slide (Menzel-Gläser, Braunschweig, Germany). Skin sections were washed with PBS for 5 minutes to remove embedding medium, then incubated with 30 μl 0.2 mg/ml IgG for 60 minutes at 37°C in a humidified air incubator containing 5% CO2. After washing the sections with PBS twice, chambers were prepared as described and 500 μl of the neutrophil suspension (1×10^7 cells/ml) was placed in each chamber. Incubation of neutrophils with skin sections was performed in a humidified air containing 5% CO2 for 3 hours at 37°C. Subsequently, chambers were disassembled, sections were washed in PBS, fixed with formalin, and subsequently stained with hematoxylin and eosin. Skin dermal-epidermal separation was evaluated by light-microscopy, and extend of dermal-epidermal separation was analyzed in a blinded fashion.

Antibody-binding Assay

The capacity of rabbit anti-mCOL7 to bind its antigen was tested by indirect immunofluorescence (IF) staining of sections (6 μm) derived from healthy C57BL/6 mouse skin using DTAF-donkey-anti-rabbit IgG (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) as detection antibody. Staining intensity of immuno-reactants at the DEJ was quantified with ImageJ software (http://rsbweb.nih.gov/ij/). Alternatively, binding of the antibodies to immobilized mCOL-7 (1 μg) was quantified by solid-phase ELISA using POD-goat-anti-rabbit IgG (Jackson ImmunoResearch Laboratory, West Grove, PA, USA) for detection.

Activation of Neutrophils in vitro

Activation of neutrophils in vitro by immobilized IC was performed as described previously with modification [13]. Briefly, mCOL7 (1 μg/ml) was coated to the bottom of a 96-well plate. After washing and blocking with PBS supplemented with 1% low endotoxin BSA and 0.05% Tween-20, the coated mCOL7 was incubated with 100 μg/ml rabbit anti-mCOL7 IgG in PBS. After removal of unbound antibodies, generation of reactive oxygen species by neutrophils was determined by measurement of chemiluminescence in the presence of 60 μg/ml luminol (5-amino-2,3-dihydro-1,4-phthalazindione; Roche Diagnostics, Mannheim, Germany). Degranulation was determined by the amount of lactoferrin and elastase released [15]. Morphology of neutrophils was monitored by light microscopy following 1 h of stimulation.

Immune Complex Binding Assay

Binding of immune complexes to neutrophils was tested by flow cytometry. Briefly, 5×10^5 neutrophils were incubated at 4°C with suspended insoluble IC (prepared with 1 μg mCOL7 and 100 μg rabbit-anti-mCOL7 IgG or EndoS-treated anti-mCOL7 IgG) for 30 min. Cell-bound IC were detected by staining with FITC-conjugated donkey-anti-rabbit IgG at 4°C.

Statistical Analysis

Data are presented as mean ± s.d. for the number of samples indicated in the figure or figure legends. Statistically significant (P<0.05) difference among the groups were calculated using one-way analysis of variance (ANOVA) test.

Ethics Statement

Approval for these studies was obtained from the Institutional Review board at the University of Lübeck (Lübeck, Germany; Az. 12-202A) according to the Declaration of Helsinki. All volunteers gave written informed consent.

Isolation of Neutrophils

Neutrophils were isolated from citrated blood of healthy donors by dextran sedimentation (Plasmagerit; Fresenius, Oberursel, Germany) followed by pancoll (PanBiotech, Aidenbach, Germany) density centrifugation [15]. More than 98% of the cells were viable as assessed by trypan blue exclusion and the percentage of neutrophils exceeded 97% in all experiments as determined by trypan blue exclusion and the percentage of eosinophils as the major contaminant. Cells were suspended in CL-medium (RPMI 1640 buffered with 25 mM HEPES without phenol red; Biochrom, Berlin, Germany) before use.

Figure 1. Effect of EndoS treatment of anti-mCOL7 IgG on tissue damage in an ex vivo model of EBA. Mouse skin cryosections were incubated with 0.2 mg/ml rabbit-anti-mCOL7 IgG (a), or EndoS-treated 0.2 mg/ml rabbit-anti-mCOL7 IgG (b) or rabbit control IgG (c) for 1 hour at 37°C. Subsequently, specimen were exposed to freshly isolated human neutrophils. Sections of a representative experiment are shown. Arrows indicate the dermal-epidermal separation. Bar = 50 μm. Furthermore, skin separation was quantified as percentage of the length of epidermis detachment in relation to the length of the total dermal-epidermal zone (d). Data are presented as mean ± s.d. of 4 independent experiments. **indicates statistically significant differences (P<0.01) between EndoS-treated and untreated IgG.

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Results and Discussion

EndoS Treatment Reduces the Pathogenicity of the Rabbit Anti-mCOL7 IgG in the ex vivo Model of EBA

In previous work we could show that EndoS treatment reduces the pathogenicity of rabbit anti-mCOL7 IgG both, in in vivo and in ex vivo models of the disease [7]. However, neither the precise deactivating principle of EndoS treatment has been elucidated so far nor could the cell population, which is functionally modulated by the modified antibodies, clearly be identified. Since the mouse skin cryosections in the ex vivo system were loaded with anti-mCOL7 IgG followed by administration of whole leukocytes, it is unclear whether only neutrophil functions are affected by EndoS treatment of the IgG, or whether its reduced pathogenicity also depends on the modulations of other cell types like macrophages or T cells. To optimize the ex vivo system, the whole leukocyte population was replaced by highly purified neutrophils and exposed to cryosections sensitized with normal rabbit anti-
mCOL7 IgG or corresponding IgG pretreated with EndoS. Incubation with rabbit anti-mCOL7 IgG in this modified ex vivo system could induce the 20±1.5% dermal-epidermal separation, while EndoS-treated rabbit anti-mCOL7 IgG only induced 0.75±1.5% separation (Figure 1). Although it has previously been described that neutrophils are necessary for the induction of dermal-epidermal separation in the ex vivo system [12], here we can show that neutrophils are also sufficient for the induction of dermal-epidermal separation in the ex vivo system. Furthermore, the significant reduction in pathogenicity of the EndoS-treated antibody as compared to the untreated controls is mediated predominantly by a modulation of neutrophil functions.

EndoS Treatment does not Alter Antigen Binding of the Rabbit Anti-mCOL7 IgG

Due to the simplicity of the ex vivo model system, the mechanisms how EndoS reduces the pathogenicity of the IgG can be narrowed down to two basic principles, a loss of binding of
the antibody to the antigen or a decreased capacity of the immune complex to activate the neutrophils. In a first step we investigated how EndoS treatment affects the binding of the antibody to the mouse COL7 within the dermal-epidermal junction on cryosections of the skin using indirect immunofluorescence-staining. While at low concentrations of the antibodies (0.01 mg/ml) no difference in binding between EndoS-treated or untreated antibodies could be observed (Figure 2e, f, g), at high concentrations (0.2 mg/ml) the binding of EndoS-treated IgG was even slightly stronger than that of untreated IgG (Figure 2b, c, g). These results indicate that EndoS treatment does not impair antigen binding of the antibody. Moreover, our findings could be confirmed in a solid-phase ELISA with immobilized recombinant mCOL7 using ELISA. Consistent with the results in immune histology binding of EndoS-treated IgG at high concentrations (>500 ng/ml) was slightly stronger than that of untreated IgG, while this difference disappeared at lower concentrations of the antibodies (Figure 2h).

Since the EndoS-treatment only affects the Fc portion of the antibody, we hypothesize that the observed increased binding signal in samples containing EndoS-treated anti-mCOL7 IgG as compared to non-treated anti-COL7 (Figure 2h) may not be due to a difference in the antigen binding of the two antibodies but rather to a preference of the detecting secondary antibody for EndoS-treated IgG. To verify this hypothesis, we coated plastic surfaces with increasing amounts of anti-mCOL7 IgG or EndoS-treated anti-mCOL7 IgG followed by incubation with a constant concentration of the FITC-conjugated secondary antibody. No differences in binding of the second antibody to treated or untreated IgG could be detected at concentrations up to 50 ng of the coated antibodies. However, at a high dosage of the coated antibodies (500 ng), a slight but significant higher binding of the second antibody to the EndoS-treated anti-COL7 IgG than to the untreated anti-mCOL7 IgG was observed (Figure S1). Our data confirm and extend findings of a previous study in vivo showing that EndoS-treatment neither affects the antibody binding nor affect the complement component deposition [7].

Figure 3. EndoS treatment of rabbit anti-mCOL7 IgG decreases neutrophil activation by immobilized immune complexes in vitro. Neutrophils were exposed for 1 hour to uncoated surfaces (control) or surfaces coated with IC generated from coated mCOL7 and rabbit anti-mCOL7 IgG (IC) or EndoS-treated rabbit anti-mCOL7 IgG (IC_EndoS). ROS release was quantified by chemiluminescence in the presence of luminol. Data of one representative experiment (a) are shown or given as mean ± s.d. of the integrals over one hour of 3 independent experiments (b). Neutrophil degranulation was determined by the amount of elastase (c) and lactoferrin released (d). Released proteins were determined in supernatants after 1 hour stimulation and given as percentage of their respective total amount. Data are presented as mean ± s.d. n=3, with statically significant differences indicated by asterisks (*p<0.05 and **p<0.001). Morphology of neutrophils (e–g) was analyzed by phase-contrast microscopy 1 hour after the stimulation. Data of one representative experiment out of 3 are given.

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EndoS Treatment Affects the Capacity of Immune Complexes to Activate Neutrophils in vitro

In a second step we investigated whether EndoS-treatment modulates the ability of immune complexes to activate neutrophils by the use of an in vitro neutrophil activation model [13]. Here, neutrophils are activated by immobilized immune complex (IC) and cell activation was determined as release of reactive oxygen metabolites (ROS), degranulation, and changes in cell morphology. Neutrophils exposed to IC generated from EndoS-treated rabbit anti-mCOL7 antibodies to FcγR on human neutrophils. This receptor expression is modulated after EndoS treatment. For this purpose, we first prepared insoluble IC and EndoS-IC. It should be noted that we observed no differences between EndoS-treated and untreated antibodies in their capacity to precipitate soluble mCOL7 (Figure S2). Washed and suspended IC or IC_EndoS were incubated at identical concentrations with neutrophils at 4°C. Bound IC were detected by flow cytometry using fluorescence conjugated secondary antibodies against rabbit IgG. While 69.1% of the neutrophils were able to bind the untreated IC in a broad peak (Figure 4a,c), only 14.8% of the cells stained positive for IC_EndoS (Figure 4b,c). However, no binding of the secondary antibody could be detected in the absence of immune complexes. Since the FITC-conjugated secondary antibody at the concentration used binds to IC and IC_EndoS in a comparable manner (Figure S3), the decrease of the fluorescence signal on IC_EndoS-treated cells cannot be explained by a reduced binding capacity of the detecting antibody. Therefore, the dramatic reduction in the binding of IC_EndoS as compared to untreated control IC clearly indicates that deglycosylation of IgG Fc affects their binding to the FcγR on neutrophils. A recent study systematically analyzed the interaction between ICs of different human IgG subclasses (with and without the glycan at Asn-297 through mutatation or enzyme hydrolysis) and the different FcRs expressed on CHO cells. This study shows that the Asn-297 is important for many, but not all, IC/FcγR interactions [20]. Here we could clearly show that the Asn-297 attached sugar moiety is important for the binding of rabbit anti-mCOL7 ICs to FcγR on human neutrophils, which explains the previously shown positive in vivo effects of EndoS seen in the EBA model [7] as well as the inhibition of neutrophil-driven dermal-epidermal separation seen in human skin sections as presented above. In light of the previous findings that EndoS treatment of human IgG from SLE patients does not activate neutrophils or PDCs, it is likely that also human anti-COL7 ICs will be reduced in their pathogenicity by EndoS hydrolysis.

In conclusion, we could show that EndoS treatment of rabbit anti-mCOL7 IgG reduces its pathogenicity in vitro and ex vivo by decreasing the binding of IC to FcγRs on human neutrophils. This sheds new light on the mechanisms of EndoS inhibition of pathogenicity in the anti-COL7 EBA model and could help to explain the positive effects seen in other models. Taken together, this supports further development of IgG glycan hydrolysisis, as exemplified by EndoS, as a potential treatment option in antibody mediated autoimmune diseases.

**Figure 4. EndoS treatment of IgG decreases the binding of IC to neutrophils.** Neutrophils were incubated at 4°C with suspended insoluble IC derived from untreated (a) or EndoS-treated anti-mCOL7 IgG (b) and cell-bound IC were detected by FITC-conjugated donkey-anti-rabbit IgG in flow cytometry. Representative results (a, b) or mean ± s.d. of the percentage of IC-positive cells derived from 3 independent experiments (c) are shown. * indicates statistically significant differences (p<0.001) between EndoS-treated and untreated IgG.

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**Decreased Binding of EndoS-treated Antibodies to Neutrophils**

The important role of IgG Fc glycans for optimal binding of the antibodies to FcγRs has been described earlier [18]. Furthermore, two previous studies showed that EndoS treatment affects the interaction between IgG and FcγRs [3,19]. Consequently, we investigated next whether the binding of IC to neutrophils is modulated after EndoS treatment. For this purpose, we first prepared insoluble IC and EndoS-IC. It should be noted that we observed no differences between EndoS-treated and untreated antibodies in their capacity to precipitate soluble mCOL7 (Figure S2). Washed and suspended IC or IC_EndoS were incubated at identical concentrations with neutrophils at 4°C. Bound IC were detected by flow cytometry using fluorescence conjugated secondary antibodies against rabbit IgG. While 69.1% of the neutrophils were able to bind the untreated IC in a broad peak (Figure 4a,c), only 14.8% of the cells stained positive for IC_EndoS (Figure 4b,c). However, no binding of the secondary antibody could be detected in the absence of immune complexes. Since the FITC-conjugated secondary antibody at the concentration used binds to IC and IC_EndoS in a comparable manner (Figure S3), the decrease of the fluorescence signal on IC_EndoS-treated cells cannot be explained by a reduced binding capacity of the detecting antibody. Therefore, the dramatic reduction in the binding of IC_EndoS as compared to untreated control IC clearly indicates that deglycosylation of IgG Fc affects their binding to the FcγR on neutrophils. A recent study systematically analyzed the interaction between ICs of different human IgG subclasses (with and without the glycan at Asn-297 through mutatation or enzyme hydrolysis) and the different FcRs expressed on CHO cells. This study shows that the Asn-297 is important for many, but not all, IC/FcγR interactions [20]. Here we could clearly show that the Asn-297 attached sugar moiety is important for the binding of rabbit anti-mCOL7 ICs to FcγR on human neutrophils, which explains the previously shown positive in vivo effects of EndoS seen in the EBA model [7] as well as the inhibition of neutrophil-driven dermal-epidermal separation seen in human skin sections as presented above. In light of the previous findings that EndoS treatment of human IgG from SLE patients does not activate neutrophils or PDCs, it is likely that also human anti-COL7 ICs will be reduced in their pathogenicity by EndoS hydrolysis.

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Supporting Information

Figure S1 Binding of FITC-conjugated donkey-anti-rabbit IgG to increasing concentrations of immobilized rabbit-anti-mCOL7 IgG or EndoS-rabbit-anti-mCOL7 IgG. Anti-mCOL7 IgG or EndoS-anti-mCOL7 IgG was coated at the concentration indicated to 96 well plates (Black IsoPlate-96 Black, PerkinElmer). After the blocking with BSA, bound IgG was detected by FITC-donkey-anti-rabbit IgG at 37 degree for 2 hours. Insoluble IC were collected and fluorescence was determined as described in the legend to Figure 2. *, P<0.05.

Figure S2 Precipitation of soluble mCOL7 by EndoS-treated and untreated anti-mCOL7 IgG. Recombinant mCOL7 (3 μg) was incubated increasing amounts of control rabbit IgG, rabbit-anti-mCOL7 IgG or EndoS-rabbit-anti-mCOL7 IgG at 37 degree for 2 hours. Insoluble IC were collected after centrifugation, washed, resuspended and then quantified photometrically at a wavelength of λ = 280 nM.

Figure S3 Binding of increasing concentrations of FITC-conjugated donkey-anti-rabbit IgG to rabbit-anti-mCOL7 IgG and EndoS-rabbit-anti-mCOL7 IgG. Anti-mCOL7 IgG or EndoS-anti-mCOL7 IgG (10 μg/ml each) was coated to 96 well plates. After the blocking, wells were incubated with FITC-donkey-anti-rabbit IgG at concentrations indicated and fluorescence was determined as described in the legend to Figure S1. *, P<0.05.

Author Contributions
Conceived and designed the experiments: XY ES FP. Performed the experiments: XY JZ. Analyzed the data: XY DZ FP. Contributed reagents/materials/analysis tools: MC ES. Wrote the paper: XY DZ FP.

References