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Aminoguanidine downregulates expression of cytokine-induced Fas and inducible nitric oxide synthase but not cytokine-enhanced surface antigens of rat islet cells

Beate Kuttler[a,*], Antje Steveling[a], Nora Klöting[a], Olaf Morgenstern[b], Heike Wanka[a]

[a]*Institute of Pathophysiology, Ernst-Moritz-Arndt-University, Greifswald, Greifswalder Str. 11c, D-17495 Karlsburg, Germany*
[b]*Institute of Pharmaceutical Chemistry, Ernst-Moritz-Arndt-University of Greifswald, D-17489 Greifswald, Germany*

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Abstract

Autoimmune β-cell destruction occurs directly by cell-mediated cytotoxicity or indirectly by cytokines released from infiltrating lymphocytes. Cytokines (IL-1β/IFN-γ) modify or induce expression of MHC antigens and ICAM-1 on β-cells which can lead to an improved binding of T-lymphocytes to β-cells and finally to an enhanced cell-mediated cytotoxicity. Cytokines also induce Fas-expression and inducible nitric oxide synthase (iNOS) causing generation of nitric oxide (NO) which is toxic for β-cells. The iNOS inhibitor aminoguanidine (AG) delays diabetes onset, but does not reduce diabetes incidence. We wanted to know whether AG inhibits cytokine-induced expression of Fas, MHC antigens and ICAM-1 on β-cells of LEW.1W and BB/OK rat islets after culture with IL-1β/IFN-γ. NO was completely inhibited by 5.0 mmol/L AG while 0.5 mmol/L had no inhibitory effect. AG downregulated Fas-expression on the surface of β-cells. Cytokine-induced/enhanced expression of MHC class-II and ICAM-1 was not affected by any AG concentration. AG syngergistically increased cytokine-induced enhancement of MHC class-I antigen density. AG possibly blocks the indirect pathway of β-cell damage *in vivo* due to inhibition of Fas and iNOS and improves direct cell-mediated cytotoxicity due to drastic increased MHC class-I expression. Inhibition of only one pathway of β-cell destruction is not sufficient to prevent diabetes.

Keywords: Aminoguanidine; β-Cell destruction; Fas; iNOS; MHC antigens; ICAM-1

1. Introduction

NO exhibits a great variety of physiological actions. NO is involved in immune defense, blood pressure regulation, inhibition of platelet aggregation and other physiological processes [1]. Therefore, it is not surprising that NO was found to play a crucial role in the destruction of pancreatic β-cells leading finally to insulin-dependent type I diabetes [2]. Even in islet transplantation formation of NO is expected to contribute to graft failure [3–5].

Acute generation of NO results from activation of the iNOS by e.g. cytokines. NO seems to be a mediator of cytokine-mediated islet toxicity [6]. During inflammation of the islets (insulitis) a variety of cytokines are released by infiltrating mononuclear cells, among them IL-1β. Intraislet release of IL-1 inhibits β-cell function by NO [7]. *In vitro*, it was impressively shown that IL-1β induces NO production in islets [8–10]. IFN-γ and TNF-α increase the sensitivity of islets for iNOS expression induced by IL-1β [11,12]. The effects of generated NO include inhibition of insulin secretion, Fas-expression (CD95) on the surface of islet cells, DNA fragmentation and subsequent cell death [7,13–20].

Generation of NO can be prevented by inhibition of NOS, which exists in two different types [21]: a constitutive isoform [cNOS found in neurons (nNOS) or in endothelial cells (eNOS)], and an iNOS. Inhibitors of NOS as L-N^G-monomethyl-arginine (L-NMMA), N^G-nitro-L-arginine, *N*-nitro-L-arginine-methylester (L-NAME), AG, nicotinamide [1,22–24] differ markedly in their effects on the various

* Corresponding author. Tel.: +49-3834-8619171; fax: +49-3834-8680117.
E-mail address: kuttler@uni-greifswald.de (B. Kuttler).

Abbreviations: AG, aminoguanidine; NO, nitric oxide; iNOS, inducible nitric oxide synthase; IL-1β, interleukin-1 beta; IFN-γ, interferon-gamma; MHC, major histocompatibility complex; ICAM-1, intercellular adhesion molecule-1; FITC, fluoresceinisothiocyanate; PE, phycoerythrine; PI, propidium iodide; LEW.1W, Lewis.1W; BB/OK, Biobreeding/Ottawa-Karlsburg.

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NOS types. Whereas AG and L-NMMA are very effective in inhibiting iNOS, L-NAME and N^G-nitro-L-arginine are less effective. Conversely, AG is less potent in the inhibition of eNOS, while L-NAME and N^G-nitro-L-arginine are very good eNOS inhibitors [1,22]. There is no doubt that AG in appropriate concentrations also inhibits eNOS and nNOS [23]. However, AG is approximately 40-fold more effective in inhibiting the inducible isoform of nitric oxide synthase [25], suggesting that AG is a selective inhibitor of iNOS. The addition of AG to islets cultured in the presence of IL-1β completely inhibits generation of NO [26]. AG is, therefore, also able to reduce the inhibitory effect of IL-1β on glucose-stimulated insulin secretion [26]. In concentrations that inhibit NO production AG is not toxic to islet cells as shown in a 6-day islet culture [27].

Besides generation of NO and upregulation of Fas-expression cytokines as IL-1β and IFN-γ induce changes in antigen expression on islet cells as increase of MHC class-I antigen density, induction of MHC class-II and increase of β-cells expressing the ICAM-1 [28]. Up to now, it is not known whether AG is also able to inhibit these cytokine-induced changes. Therefore, the aim of the present study was to examine whether AG also inhibits cytokine-induced Fas-expression and cytokine-induced changes of antigen expression on β-cells of LEW.1W and BB/OK rat islets.

2. Material and methods

2.1. Animals

All rats used in this study were bred in the Animal Laboratories of our institute. They were kept under specific pathogen-free conditions and had free access to standard chow pellets and sterilized water. Diabetes-resistant LEW.1W (RT.1^u) and diabetes-prone BB/OK (RT1^u) rats were used at an age of 8–12 days for preparation of pancreatic islets.

2.2. Preparation of isolated islets of Langerhans

Pancreatic islets were prepared by fractionated collagenase digestion (Serva, 0.6 mg/mL). Isolated islets were separated from exocrine tissue by centrifugation on a dextran gradient and further purified by hand-picking [29].

2.3. Cytokine culture

Groups of 500 freshly-isolated islets were cultured in 24-well Linbro® plates (ICN Biochemicals) in 0.5 mL RPMI 1640 (ICN Biochemicals) containing 10% FCS, 11 mmol/L glucose, 5 mmol/L L-glutamine, 100 IE/mL penicillin and 100 μg/mL streptomycin. The islets were either cultured (1) in medium (control), (2) in the presence of recombinant human IL-1β (1.0 ng/mL) plus rat interferon-γ (rIFN-γ, 100 IU/mL, kindly provided by Dr. P.H. van der Meide, BPRC-TNO, Rijswijk, The Netherlands), (3) in the presence of AG (5.0 mmol/L) or (4) in the presence of IL-1β plus IFN-γ plus AG (0.5 or 5.0 mmol/L). The culture was performed for 48 hr at 37° and 95% O_2/5% CO_2.

After the culture supernatants were collected for assay of nitrite levels and for determination of insulin accumulation (see below). Duplicates of five islets each were taken for measurements of islet insulin content. Additionally, islets were taken to prepare single cells for determination of antigen expression (see below).

2.4. Measurement of NO as nitrite

Nitrite, the stable product of NO in aqueous solution, was determined by the Griess reaction [30] using the Reagent System of Promega. The assay relies on a diazotization reaction. Culture media samples (50 μL) were mixed with an equal volume of the sulfanilamide solution (1% in 5% H_3PO_4). After incubation for 10 min at room temperature 50 μL of 0.1% naphthylethylene diamine dihydrochloride (NED) were added. The incubation with sulfanilamide solution and NED was done in two different steps, since sulfanilamide and NED compete for nitrite in the Griess reaction, thus greater sensitivity is achieved when the two compounds are added sequentially. The absorbance was measured at 520 nm on the plate reader Anthos ht II photometer (Anthos Labtec Instruments). The concentration of nitrite was determined by means of a sodium nitrite standard curve. All conditions in each experiment were run in the same assay.

2.5. Measurement of islet insulin content and medium insulin accumulation

After the 48 hr culture duplicates of five islets of each condition were homogenized by ultrasonication in an acidified solution. Islet insulin content and insulin accumulation in the culture medium were measured by ELISA (Mercodia) and calculated in pmol/islet.

2.6. Preparation of single islet cells

Islets were dispersed into single cells by fractionated digestion with dispase II (Roche) after an incubation of islets for 10 min at room temperature in Ca^{2+}-free Hanks balanced salt solution [31]. Cell number was counted using a Neubauer chamber. Viability of single islet cells was determined by trypan blue exclusion [32]. Viability of control islet cells was >95%. Single cells obtained from cytokine-cultured islets had a viability of about 70%.

2.7. Antigen expression on single islet cells

Antigen expression of islet cells was determined by flow cytometry using the double-staining technique [33]. The

cells were stained with monoclonal antibodies recognizing MHC class-I antigens [OX18-PE], MHC class-II antigens [OX6-PE] and the ICAM-1 (CD54) [1A29-PE]. All three antibodies were purchased from Pharmingen. The proportion of pancreatic β-cells was determined by using the FITC-conjugated monoclonal antibody K14D10 recognizing only β-cells [34,35]. The rabbit anti-Fas polyclonal antibody (StressGen Biotechnologies Corp) was used to determine the Fas expression. The binding of this antibody was visualized by FITC-conjugated AffiniPure $F(ab')_2$ fragment donkey anti-rabbit IgG (H + L) (Dianova).

A minimum of 0.5×10^5 cells were incubated with the antibodies for 15 min at 4° in the dark in a final volume of 70 μL phosphate buffered saline containing 0.1% NaN_3 and 10% FCS (PBS). Cells which were stained with the unlabeled anti-Fas antibody were washed with 4 mL PBS and incubated with anti-rabbit IgG-FITC. After final washing with 4 mL PBS stained cells were analysed on a FACS Calibur (BD) by means of an air-cooled 488 nm 15 mW argon laser and detectors for forward scatter, 90° light scatter (side scatter), fluorescence 1 (FL1: FITC = green) and fluorescence 2 (FL2: PE = orange). Fas-stained islet cells were additionally stained with 2.5 μL PI (0.5 μg/mL) 5 min before the cells were analysed.

The results were expressed as percentage of cells which were stained positive. Furthermore, we registered antigen density as mean fluorescence intensity of a staining in logU.

2.8. Statistical analysis

The results of N experiments are given in mean ± SEM and the ANOVA-test (Bonferroni's multiple comparison test) was used to check statistical significance.

3. Results

3.1. Cumulative insulin release into the medium and islet insulin content

During the 48 hr culture period of LEW.1W rat islets in the presence of cytokines cumulative release of insulin into the medium was significantly decreased (Table 1). Whereas the addition of 5.0 mmol/L AG reversed this effect ($P < 0.01$) the addition of 0.5 mmol/L AG was not able to normalize insulin release into the medium (Table 1). Although there is a statistically significant reduction of cumulative insulin release from BB/OK rat islets, the effect did not differ from that of AG alone and addition of 5.0 mmol/L AG was not able to reverse this effect (Table 1). AG alone had no significant effect on insulin release into the medium using LEW.1W rats.

Insulin content of LEW.1W control islets amounted to 4.22 ± 0.41 pmol/islet, which was significantly decreased after culture in the presence of cytokines (Table 1). Addition of 5.0 mmol/L AG to the LEW.1W-islets-cytokine-culture increased islet insulin content which was no longer significantly different compared to LEW.1W control islets (Table 1). Islet insulin content of cytokine-exposed LEW.1W rat islets was not normalized using AG in the lower concentration. BB/OK rat control islets had an insulin content of 5.24 ± 0.45 pmol/islet. Cytokines reduced this value significantly to 3.03 ± 0.35 pmol/islet (Table 1). Addition of AG to the BB/OK-islet-cytokine-culture was not effective concerning recovery of insulin content (Table 1). Again, AG alone had no effect on islet insulin content in both rat strains investigated (Table 1).

Table 1
Cumulative insulin release by isolated islets during culture and insulin content of islets after the culture

	LEW.1W rat islets	BB/OK rat islets
Cumulative insulin release (pmol/islet × 2*d*)		
N	10	6
Control	1.92 ± 0.15	1.89 ± 0.34
AG (5.0 mmol/L)	1.61 ± 0.17	1.15 ± 0.16
IL-1β + IFN-γ	0.74 ± 0.13$^{***,\#\#\#,++}$	1.03 ± 0.13*
IL-1β + IFN-γ + AG (5.0 mmol/L)	1.71 ± 0.19	1.20 ± 0.20
IL-1β + IFN-γ + AG (0.5 mmol/L)	0.70 ± 0.11$^{***,\#\#\#,++}$	n.d.
Islet insulin content (pmol/islet)		
N	10	8
Control	4.22 ± 0.41	5.24 ± 0.45
AG (5.0 mmol/L)	4.21 ± 0.35	4.79 ± 0.48
IL-1β + IFN-γ	2.43 ± 0.27$^{**,\#}$	3.03 ± 0.35$^{**,\#}$
IL-1β + IFN-γ + AG (5.0 mmol/L)	3.45 ± 0.35	3.53 ± 0.36*
IL-1β + IFN-γ + AG (0.5 mmol/L)	2.67 ± 0.22*	n.d.

The islets obtained from LEW.1W and BB/OK rats were cultured for 48 hr in the presence of AG, IL-1β in combination with IFN-γ or in the presence of both cytokines and AG. For details see Section 2. n.d.: not determined.

*** $P < 0.001$ vs. control; ** $P < 0.01$ vs. control; * $P < 0.05$ vs. control; $^{\#\#\#}$ $P < 0.001$ vs. AG (5 mmol/L); $^{\#}$ $P < 0.05$ vs. AG (5 mmol/L); $^{++}$ $P < 0.01$ vs. IL-1β + IFN-γ + AG (5.0 mmol/L).

3.2. NO production

Islets isolated from LEW.1W rats or from BB/OK rats produced equal amounts of NO measured as nitrite in response to IL-1β and IFN-γ (Table 2). As expected aminoguanidine inhibited cytokine-induced production of NO released by BB/OK or LEW.1W rat islets (Table 2). This inhibition was complete by using AG at a concentration of 5.0 mmol/L. If AG was used at a concentration of 0.5 mmol/L there was no inhibition of NO produced by LEW.1W rat islets.

3.3. Surface Fas-expression (CD95)

Spontaneous Fas-expression of islet cells from 48 hr-cultured LEW.1W and BB/OK rat islets was registered

Table 2
NO production by isolated LEW.1W and BB/OK rat islets after stimulation with the cytokines IL-1β in combination with IFN-γ and inhibition of NO production by AG

	LEW.1W rats (pmol/islet)	BB/OK rats (pmol/islet)
N	9	8
Control	1.3 ± 0.3	1.1 ± 0.3
AG (5.0 mmol/L)	0.5 ± 0.3	0.4 ± 0.2
IL-1β + IFN-γ	27.0 ± 1.5$^{***,\#\#\#,+++}$	23.4 ± 1.3$^{***,\#\#\#,+++}$
IL-1β + IFN-γ + AG (5.0 mmol/L)	1.1 ± 0.3	0.8 ± 0.2
IL-1β + IFN-γ + AG (0.5 mmol/L)	24.0 ± 3.8$^{***,\#\#\#,+++}$	n.d.

The stimulation was carried out for 48 hr. For details see Section 2. n.d.: not determined.
*** $P < 0.001$ vs. control; $^{\#\#\#}$ $P < 0.001$ AG (5 mmol/L); $^{+++}$ $P < 0.001$ vs. IL-1β + IFN-γ + AG (5.0 mmol/L).

with 6–8%. This expression was not influenced by AG (5.0 mmol/L). Cytokines upregulated Fas significantly to about 25%, corresponding to a 3–4-fold increase. This is demonstrated by representative histograms in Fig. 1 for LEW.1W islet cells.

Staining of cytokine-cultured islet cells with PI revealed three populations (Fig. 2A): (1) PI negative islet cells (about 35%), (2) PI positive islet cells with low PI staining density (MFI $< 10^2$ logU, about 25%) and (3) PI positive islet cells with high PI staining density (MFI $> 10^2$ logU, about 40%). Analysing cytokine-induced Fas expression in more detail by co-staining of PI, it became evident that there were two Fas^+ cell populations with different PI expression (Fig. 2B and C): we found $PI^{low}Fas^+$ islet cells (24.2%) and $PI^{high}Fas^+$ islet cells (7.2%, Fig. 2C). There were no Fas^+ islet cells which were PI negative. The $PI^{low}Fas^+$ cell population could already be identified in the dot plot histogram in which the cells were only stained for PI (Fig. 2B, 25.1% of islet cells within the circle vs. 24.2% within the circle in Fig. 2C). It was also evident that nearly all (>95%) PI^{low} islet cells were Fas positive (24.2% of 25.1%), while about 80% of the PI^{high} islet cells remained Fas negative (30.5% of 39.9%).

Aminoguanidine markedly ($P < 0.001$) reduced cytokine-induced Fas-expression on β-cells of LEW.1W rats (Fig. 1) and BB/OK rats. However, this inhibition was not complete. If one equate Fas-expression induced by

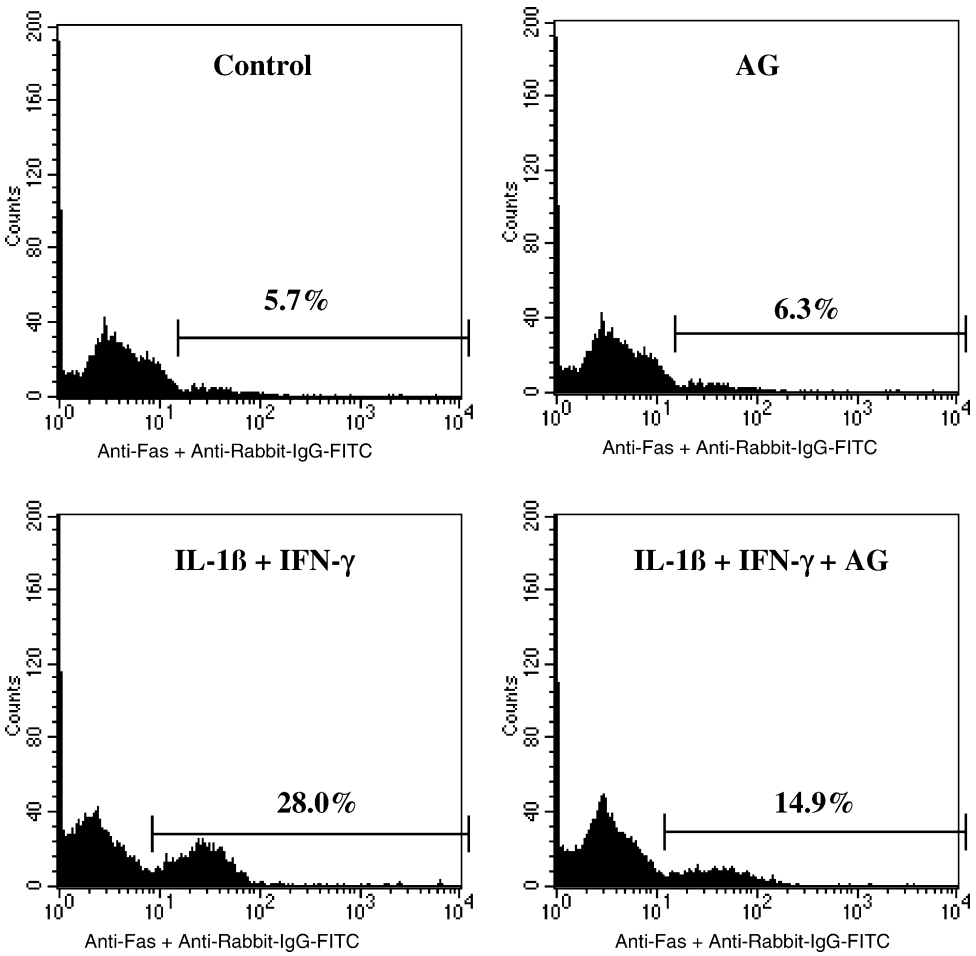

Fig. 1. Representative one-parameters histograms of islet cells from LEW.1W rats stained with anti-Fas and FITC-labeled anti-rabbit-IgG. Prior to staining procedure islets were cultured for 48 hr either alone (control), in the presence of AG (5.0 mmol/L), cytokines (IL-1β + IFN-γ) or in the presence of cytokines plus AG. For details see Section 2.

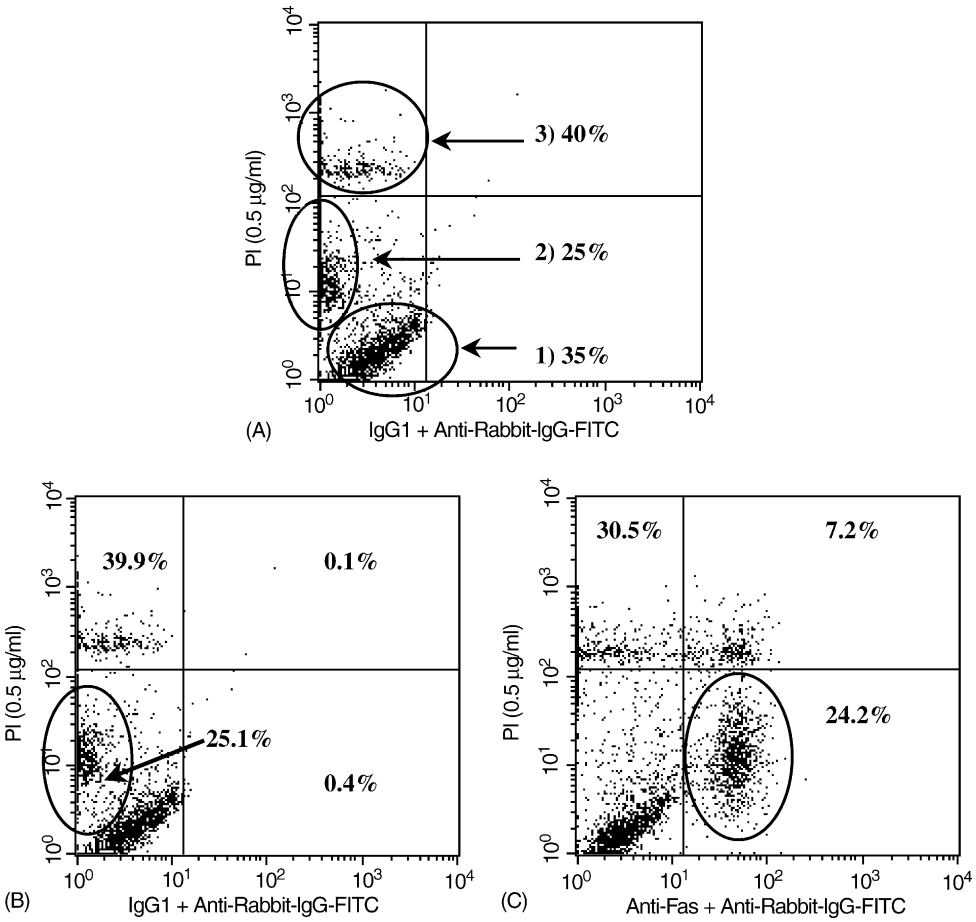

Fig. 2. Representative dot plot histograms of islet cells from LEW.1W rats stained (A) and (B) with PI (0.5 µg/mL) and a negative control antibody and with FITC-labeled anti-rabbit-IgG and (C) with PI (0.5 µg/mL) and anti-Fas/FITC-labeled anti-rabbit-IgG. Prior to staining procedure islets were cultured for 48 hr in the presence of IL-1β and IFN-γ. (A) In dependence on PI staining the circles characterize three different cell populations: (1) PI negative islet cells, (2) PI positive islet cells with low PI staining density (MFI $< 10^2$ logU) and (3) PI positive islet cells with high PI staining density (MFI $> 10^2$ logU). (B) Cells which were only stained with PI demonstrate a population of 25.1% with low PI density (see cells within the circle), these cells become Fas positive as demonstrated in (C). (C) Most of the Fas positive islet cells have a PI staining with a low density (see cells within the circle) which are possibly apoptotic cells. Fas positive islet cells with a high density PI staining (7.2%) are thought to be necrotic. For details see Section 2.

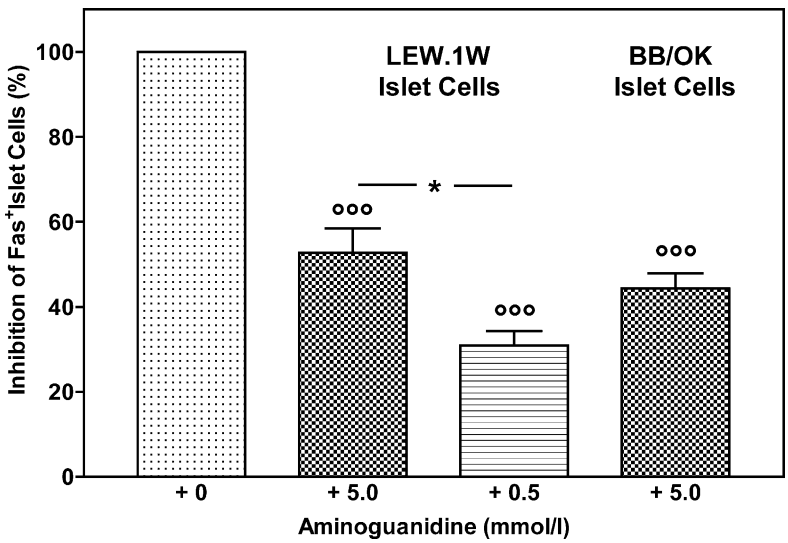

Fig. 3. Inhibition of Fas-expression on islet cells of LEW.1W (N = 11) and BB/OK rats (N = 7) by AG. Islets were cultured for 48 hr in the presence IL-1β plus IFN-γ (=100%, ⬚). For inhibition of Fas-expression AG was added to the culture in two different concentrations (5.0 mmol/L, ▩ and 0.5 mmol/L, ▤). For details see Section 2. °°°$P < 0.001$ vs. culture with IL-1β plus IFN-γ but without AG (left column; +0), * $P < 0.05$.

cytokines to 100%, addition of 5.0 mmol/L AG reduced it to 52.7 and 44.3% on LEW.1W rat islet cells and BB/OK rat islet cells, respectively (Fig. 3). In case of LEW.1W rat islets Fas-expression was further reduced to 30.9% by addition of 0.5 mmol/L AG. Inhibition of cytokine-induced Fas-expression by 5.0 mmol/L AG was not significantly different between LEW.1W and BB/OK rat islets.

Additionally, we analysed the capacity of AG to reduce the proportion of cytokine-induced $PI^{low}Fas^{+}$ islet cells compared to the capacity to reduce the proportion of cytokine-induced $PI^{high}Fas^{+}$ islet cells. In case of islet cells from LEW.1W rats it was apparent that the $PI^{low}Fas^{+}$ population was significantly ($P < 0.001$) inhibited by 5 mmol/L AG whereas the $PI^{high}Fas^{+}$ population was not (Fig. 4). The lower concentration of AG caused a further reduction of the $PI^{low}Fas^{+}$ population and reduced also $PI^{high}Fas^{+}$ cells, although these effects were not statistically significant (Fig. 4). Conversely, both islet cell populations from BB/OK rats were significantly reduced by AG, however, the reduction of $PI^{low}Fas^{+}$ islet cells was much more pronounced (Fig. 4).

3.4. Antigen expression on single islet cells

Pancreatic β-cells cells do not express MHC class-II antigens on their surface. AG (5.0 mmol/L) alone did not induce MHC class-II on islet cells (Fig. 5). Induction of MHC class-II antigen expression was observed after culture of LEW.1W or BB/OK rat islets in the presence of IFN-γ and IL-1 (Fig. 5). Addition of AG (5.0 mmol/L) to the cytokine culture had no effect on cytokine-induced MHC class-II antigen expression.

More than 98% of islet β-cells express MHC class-I molecules on their surface which was not altered by any

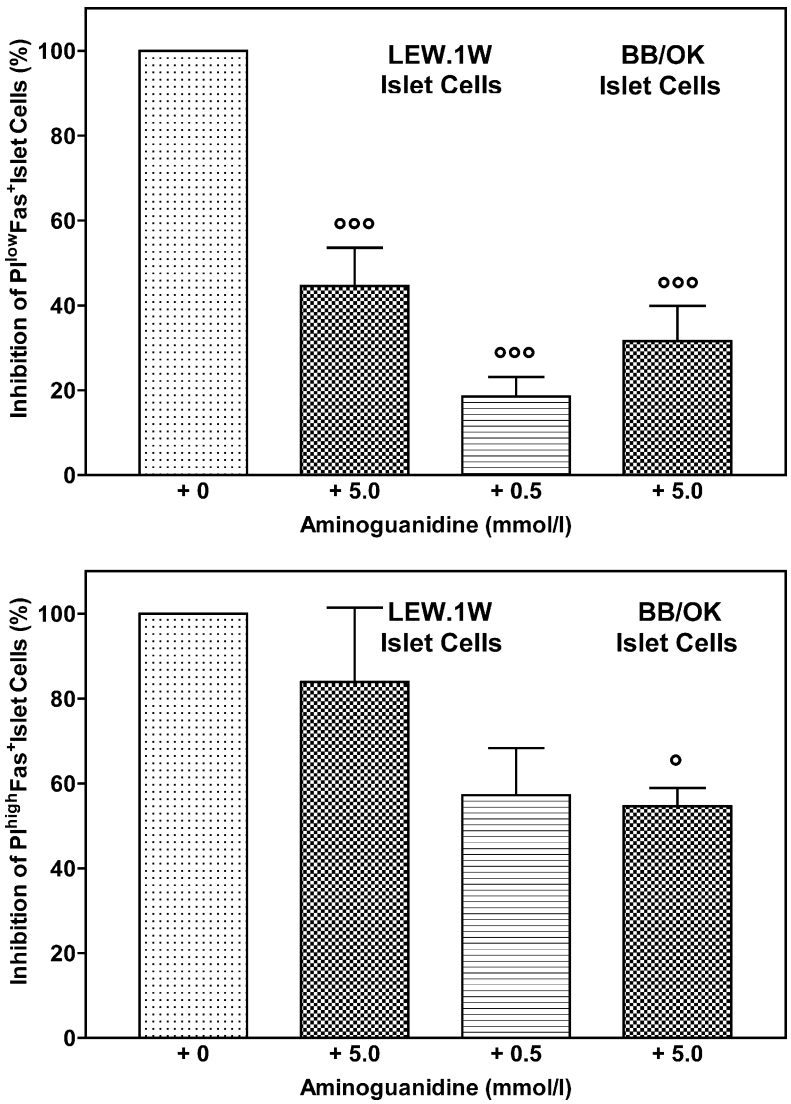

Fig. 4. Inhibition of $PI^{low}Fas^{+}$- and $PI^{high}Fas^{+}$-cells of LEW.1W (N = 7) and BB/OK rats (N = 7) by AG. Islets were cultured for 48 hr in the presence IL-1β plus IFN-γ (=100%, ⬚). For inhibition of $PI^{low}Fas^{+}$- and $PI^{high}Fas^{+}$-cells AG was added to the culture in two different concentrations (5.0 mmol/L, ▩ and 0.5 mmol/L, ▤). For details see Section 2. $^{o}P < 0.05$, $^{ooo}P < 0.001$ vs. culture with IL-1β plus IFN-γ but without AG (left column; +0).

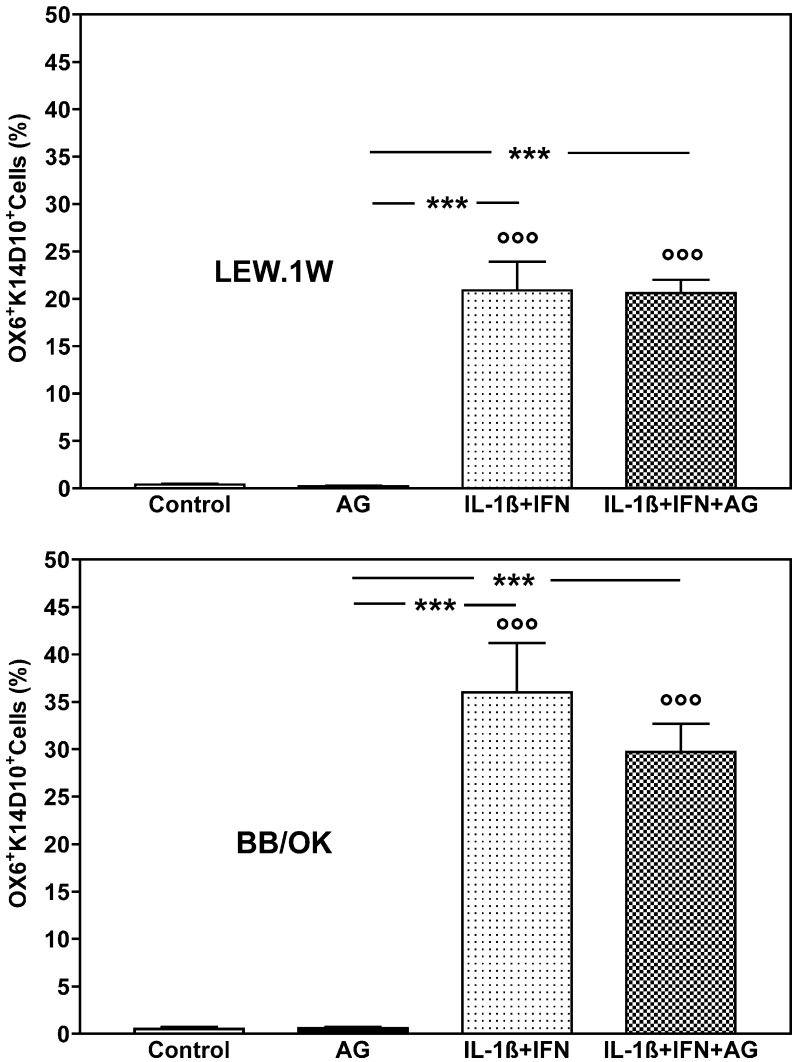

Fig. 5. MHC class-II antigen expression on islet β-cells from LEW.1W (N = 8) and BB/OK rats (N = 6) as determined by two-color flow cytometry after culture of islets for 48 hr alone (□), in the presence of AG (5.0 mmol/L, ■), IL-1β plus IFN-γ (⬚) or in the presence of cytokines plus AG (▩). For details see Section 2. $^{ooo}P < 0.01$ vs. control; $^{***}P < 0.001$.

culture condition investigated (data not shown). However, antigen density of MHC class-I on the cell surface of β-cells was markedly enhanced by IFN-γ and IL-1 (Fig. 6). Although AG (5.0 mmol/L) alone had no effect on both cell types, it synergistically increased MHC class-I antigen density after culture of islets in combination with cytokines (Fig. 6). There was no difference between islets isolated from LEW.1W or BB/OK rats.

As shown in Fig. 7 about 25–30% of pancreatic β-cells were positive for the ICAM-1. This proportion was not significantly changed by culture of islets in the presence of AG (5.0 mmol/L) alone (Fig. 7). IFN-γ and IL-1 caused an increase of ICAM-1^+ β-cells to more than 60%. Addition of AG (5.0 mmol/L) did not influence this proportion, neither on islets cells from LEW.1W rats nor on BB/OK rat islet cells (Fig. 7).

Using AG in a concentration of 0.5 mmol/L identical results as described were obtained (data not shown). We did not observe any reduction of cytokine-induced enhancement of MHC class II^+ β-cells, MHC class I antigen density or ICAM-1^+ β-cells.

4. Discussion

Inflammation of pancreatic islets (insulitis) is seen in autoimmune diabetes long before disease manifestation occurred in humans as well as in corresponding animal models, e.g. NOD mice and BB rats. Infiltrating mononuclear cells (macrophages, T-lymphocytes) can be toxic to β-cells either by direct cell-mediated cytotoxicity or indirectly by the release of cytokines as IL-1β, TNF-α and IFN-γ. These cytokines have the potential of direct β-cell killing or they are able to induce the expression of the iNOS. This enzyme was localized within pancreatic lesions in macrophages and also in pancreatic β-cells of adult prediabetic BB rats and NOD mice [36–38]. Consequently, NO and also peroxynitrite ($ONOO^-$) are generated locally.

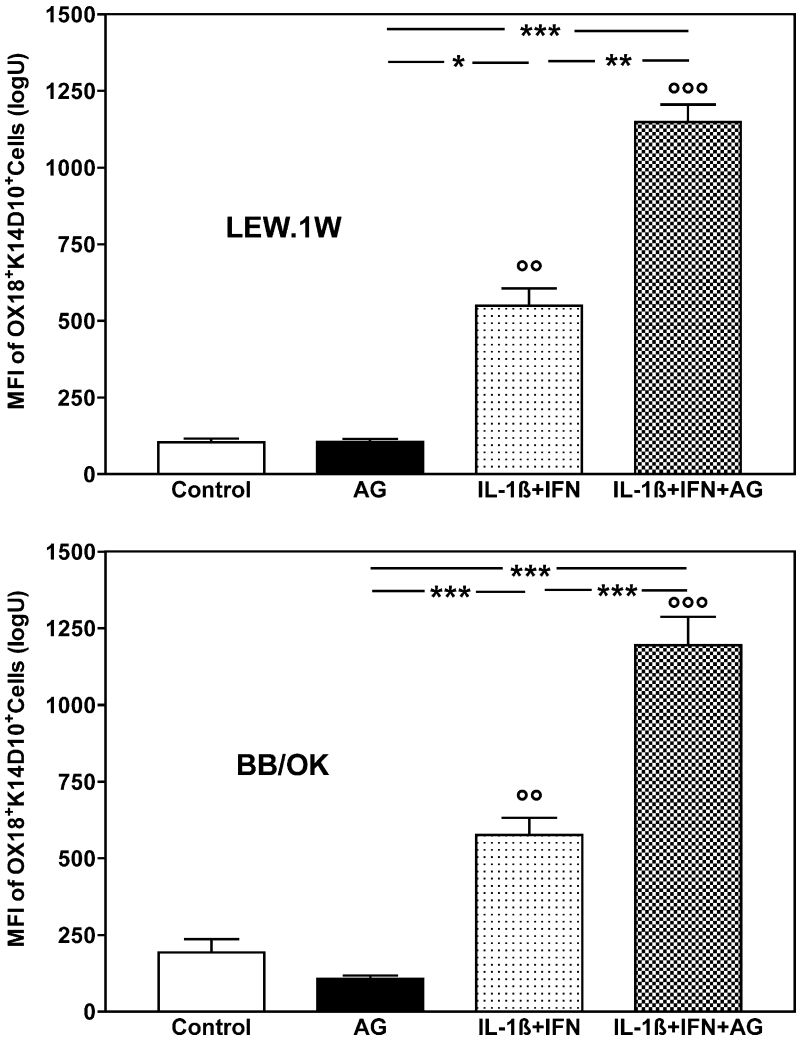

Fig. 6. MHC class-I antigen density on islet β-cells from LEW.1W (N = 7) and BB/OK rats (N = 6) as determined by two-color flow cytometry after culture of islets for 48 hr alone (□), in the presence of AG (5.0 mmol/L, ■), IL-1β plus IFN-γ (⬚) or in the presence of cytokines plus AG (▩). For details see Section 2. $^{oo}P < 0.01$, $^{ooo}P < 0.001$ vs. control; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

In vitro studies have been shown that iNOS can be inhibited completely by aminoguanidine [39–41]. Furthermore, *in vivo* application of AG attenuated endotoxin-induced uveitis in rats [42] and improved experimental autoimmune encephalomyelitis in mice [43]. Since these results underline the suggested role for NO in the development of autoimmune diseases, attempts to prevent diabetes in diabetes-prone animals have been undertaken. Unfortunately, administration of AG to both BB rats and NOD mice failed to prevent diabetes [44–46]. AG delayed the onset of diabetes without altering the rate of diabetes incidence [44,45]. In nearly all of these studies AG was applied in a concentration of 50 mg/kg body weight. Bowman *et al*. [46] reported for their NOD mice plasma levels of about 9 μg/mL 30 min after AG injection (i.p.). This concentration corresponds to about 0.1 mmol/L AG, a concentration which is less than we used in our *in vitro* experiments. Holstad *et al*. [27] showed that this concentration is sufficient for inhibiting iNOS. However, we found no inhibition of NO by using 0.5 mmol/L AG (see Table 2). In some experiments we could show that cytokine-induced Fas was still inhibited by 0.1 mmol/L AG to about 40% (data not shown).

However, a new selective inhibitor of iNOS was found to be effective *in vitro* and *in vivo*. In NOD mice guanidinoethyldisulphide (GED) delayed diabetes onset (from age 12 to 22 weeks) and significantly decreased diabetes incidence at 30 weeks from 80 to 17% [47]. Furthermore, GED delayed onset of experimental encephalomyelitis and decreased the number of animals displaying disease signs [48]. There might be additional effects of GED compared to AG which can explain the different results in preventing diabetes in NOD mice. It is reported that GED is also a scavenger for peroxynitrite which is formed by reaction of NO with superoxide. GED prevents cytokine- and peroxynitrite-induced β-cell destruction. This protective effect was related to inhibition of peroxynitrite and not to inhibition of NO, since L-NMMA as iNOS inhibitor prevented

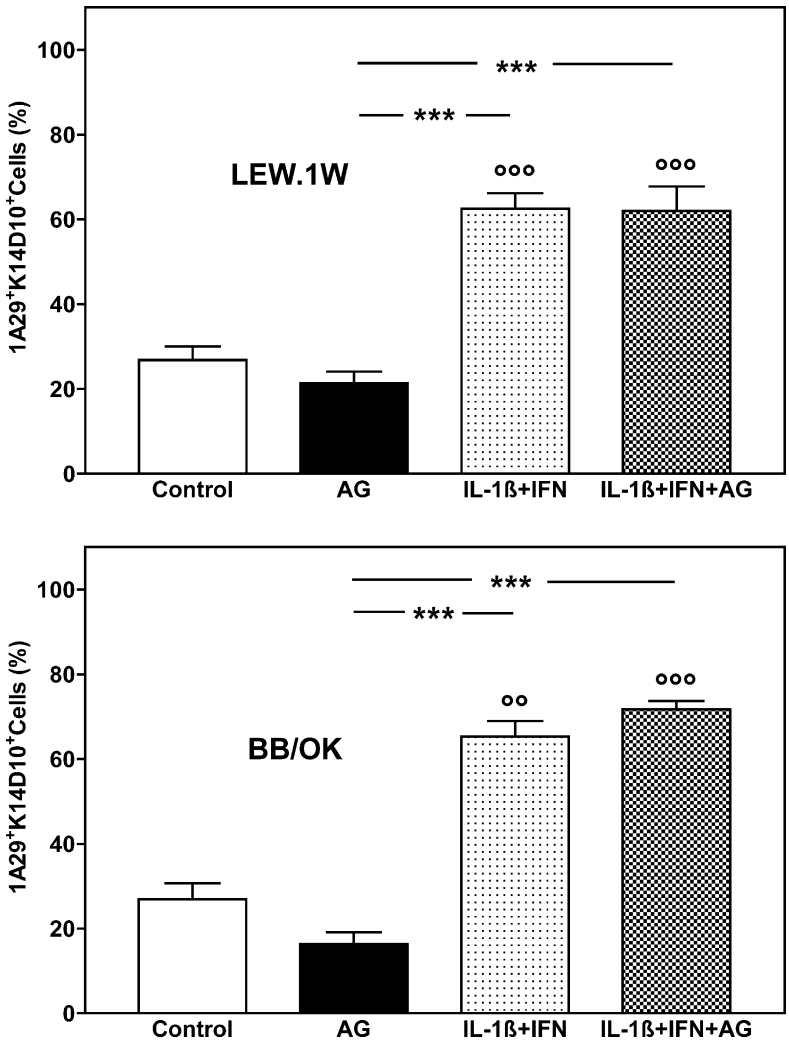

Fig. 7. ICAM-1 expression on islet β-cells from LEW.1W (N = 10) and BB/OK rats (N = 6) as determined by two-color flow cytometry after culture of islets for 48 hr alone (□), in the presence of AG (5.0 mmol/L, ■), IL-1β plus IFN-γ (⬚) or in the presence of cytokines plus AG (▩). For details see Section 2. $^{oo}P < 0.01$, $^{ooo}P < 0.001$ vs. control; $^{***}P < 0.001$.

cytokine-induced NO generation, but did not prevent cytokine-induced peroxynitrite formation and β-cell destruction [49]. Therefore, it can be assumed that also AG has no effect on peroxynitrite formation. However, we present additional results which can explain the missing effect of AG in reducing diabetes incidence.

As already reported, AG inhibited iNOS *in vitro* completely when used in an appropriate concentration. This result was absolutely confirmed by our investigations. We found a complete inhibition of cytokine-induced iNOS by 5.0 mmol/L AG, while 0.5 mmol/L were completely ineffective. Using a rat β-cell line (RIN 5AH) 0.5 mmol/L AG caused an inhibition of cytokine-induced NO of 89%. An inhibition of about 50% was found with 0.1 mmol/L AG.[1] Similar results were reported by Zhang *et al.* [50] who found that an AG concentration of 0.1 mmol/L inhibited cytokine-induced iNOS by about 50% in cultured rat hepatocytes. It seems that there is a difference in the effectiveness of AG in experiments using single cells or suborgans consisting of a lot of single cells. Islets are suborgans consisting of 3000–5000 single cells among them the insulin-producing β-cells. Consequently, much more AG is necessary to inhibit cytokine-induced iNOS in case of cultured islets.

According to the inhibition of NO production, AG caused a dose-dependent restoration of cumulative insulin release into the culture medium and islet insulin content decreased by IL-1β and INF-γ. This result implicates that NO participated really in the destruction of β-cells *in vitro*.

Fas is a 45 kDa cell surface protein belonging to the tumor necrosis factor receptor family [51,52]. This receptor is expressed on a wide variety of cells and is a dead signal for cells in Fas-mediated apoptosis [53]. Fas-mediated apoptosis seems to play a role in autoimmune destruction of pancreatic β-cells. Under physiological conditions β-cells do not express Fas on their surface [54,55]. However, in diabetic animals and also in newly diagnosed diabetic patients Fas expression is induced on β-cells in inflamed

[1] Unpublished data.

islets [55,56]. Furthermore, it was shown that cytokines are able to induce Fas on the surface of islet cells *in vitro* ([54], see results). Interaction between Fas-expressing β-cells and FasL positive activated T-lymphocytes leads to apoptotic β-cell death. In our investigations AG downregulated cytokine-induced Fas expression on the β-cell surface demonstrating that AG might be able to interfere with the Fas-dependent pathway of β-cell destruction. However, the AG concentration seems to be important. Since 0.5 mmol/L AG was more effective than 5.0 mmol/L in downregulation of Fas and since 5.0 mmol/L AG alone reduced cumulative insulin release from BB/OK rats islets markedly one can speculate that the higher concentration might be already toxic to β-cells.

Up to now it is not clear whether the cytokines itself or the cytokine-induced NO is responsible for induction of Fas expression on β-cells. Stassi *et al.* [19] demonstrated that the NO donors sodium nitroprusside and nitric oxide releasing compound-18 (NOC-18) induce functional Fas expression in pancreatic β-cells. This result suggests that NO itself is able to induce Fas expression. As expected the very potent iNOS inhibitor L-NMMA did not interfere with NO-induced Fas expression, while it inhibits IL-1β-induced Fas expression. These results are not confirmed by us and others [57,58]. Liu *et al.* [57] demonstrated that Fas mRNA expression occurred also in islets from iNOS deficient mice exposed to cytokines, indicating a cytokine-dependent but NO-independent Fas expression. In accordance with this result we found a discrepancy between the inhibitory effects of AG on NO production and on Fas expression of islet cells. Whereas the low concentration of AG (0.5 mmol/L) still effectively inhibited cytokine-induced Fas expression by about 70% it was completely ineffective in preventing NO production. Therefore, a direct role of NO in inducing Fas-mediated apoptosis of β-cells can be excluded and AG reduces cytokine-induced Fas expression not by inhibiting iNOS. Other properties of AG must be responsible for Fas downregulation. It is well known that AG does not only inhibit iNOS but also a variety of other enzymes, such as diamine oxidase, advanced glycation endproducts synthase, and proteinases [59]. It is unlikely that inhibition of proteinases by AG contribute to the reduction of cytokine-induced Fas expression, since the Fas receptor is a cell surface protein. Inhibition of proteinases should, therefore, be rather without effect on cell surface proteins. Inhibition of diamine oxidase interferes with histamine metabolism, which seems not to be involved in Fas expression. Further, IL-1β and IFN-γ induce coexpression of both iNOS and inducible cyclooxygenase (COX-2) [60,61]. The generated NO activates COX-2 [60]. *In vitro*, COX-2 is not inhibited by AG [62], it rather seems to be enhanced [63]. Since COX-2 inhibits Fas-mediated apoptosis [64] and since inhibition of COX-2 induces apoptosis [65] one could speculate that AG downregulated Fas in our experiments by regulation of COX-2. Furthermore, AG is able to inhibit RNA and DNA synthesis [66,67]. If this would be true in our islet cultures, one could explain the reduction of cytokine-induced Fas expression by such an inhibition of RNA and DNA synthesis.

A detailed analysis of Fas expressing cells by staining the cells with PI revealed two different Fas^+ cell populations, which differed markedly in the intensity of the PI staining. We recognized Fas^+ cells with high density of PI ($PI^{high}Fas^+$ cells) and with low density of PI ($PI^{low}Fas^+$ cells). PI is normally used to identify dead cells. Therefore, we speculate that Fas^+ cells which are PI^{high} are dead cells (necrotic cells or cells in the end stage of apoptosis). Thus, Fas^+ cells which are PI^{low} are just starting to die. Consequently, we hypothesize that $PI^{low}Fas^+$ cells are apoptotic cells while $PI^{high}Fas^+$ cells are necrotic cells. AG (5.0 mmol/L) was able to reduce the proportion of $PI^{low}Fas^+$ cells while the proportion of $PI^{high}Fas^+$ cells was not significantly decreased. The lower concentration of AG (0.5 mmol/L) also reduced only the proportion of $PI^{low}Fas^+$ cells significantly. From these results one can conclude (1) that β-cell destruction by the cytokine combination used is mediated by both apoptosis and necrosis, and (2) that AG is only capable of reducing apoptotic cells effectively.

As shown by our investigations IL-1β and IFN-γ enhanced MHC antigen and ICAM-1 expression. This cytokine-induced enhancement of surface antigen expression which also occurres *in vivo* during inflammation might improve the binding of cytotoxic lymphocytes to β-cells. For the first time, we demonstrated that AG was neither able to reduce cytokine-induced induction of MHC class-II antigen expression on β-cells nor cytokine-induced increase of ICAM-1 expression. On the contrary, AG further enhanced cytokine-induced increase of MHC class-I antigen density on β-cells. Therefore, we conclude that AG is not able to reduce the direct cell-mediated cytotoxicity of T-lymphocytes. More likely, AG seems to improve this way of β-cell destruction specially due to the synergistic action of the cytokines and AG in enhancing MHC class I antigen density.

Taken together, our results explain the delay of diabetes onset by AG as well as the missing effect of AG on diabetes incidence. Since AG inhibited cytokine-induced iNOS expression completely and Fas-expression to a certain extent one can speculate that the indirect pathway of β-cell damage by cytokines and NO might be reduced *in vivo*. However, on the other hand the direct contact of β-cells with β-cell specific T-lymphocytes seems to be facilitated by the drastic increase of MHC class I antigen expression. Thus, the inhibition of only one pathway of β-cell destruction, e.g. the indirect pathway, is not sufficient to prevent diabetes.

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