

Using the rat  $\beta$ -cell RIN-5AH insulinoma line as a means for studying insulin-dependent diabetes mellitus (IDDM), it is shown that interleukin-1 (IL-1) induces  $\beta$ -cell damage initiated by early apoptotic signals. This action is demonstrated by DNA fragmentation, as assessed by specific BrdU labeling, surface expression of Fas and nitric oxide (NO) production. In addition, the interplay between NO and Fas is shown, while scanning electron microscopy (SEM) confirms apoptosis by revealing the degree and type of cellular damage which, in the case of IL-1 $\alpha$ , can be reversed by an inhibitor to NO synthesis. Apoptosis is also reconfirmed by transmission electron microscopy (TEM) by observing condensed nuclear chromatin after IL-1 exposure. Thus, treatment of insulinoma cells with IL-1 $\alpha$  and IL-1 $\beta$  seems to initiate a number of signals, including PKC activation as published previously, that ultimately lead to  $\beta$ -cell destruction. Each IL-1 isoform, however, definitely follows a different pathway of action.

**Key words:** IDDM, IL-1, Apoptosis, Chromatin condensation, BrdU, Fas, Nitric oxide, PKC, Electron microscopy

## The destructive action of IL-1 $\alpha$ and IL-1 $\beta$ in IDDM is a multistage process: evidence and confirmation by apoptotic studies, induction of intermediates and electron microscopy

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## Introduction

Insulin-dependent diabetes mellitus (IDDM) is a chronic autoimmune disease characterized by specific destruction of insulin-secreting  $\beta$  cells of the pancreas.<sup>1,2</sup> Pancreatic Langerhans islets are composed of a heterogeneous population of secretory cells ( $\alpha$  and  $\beta$  cells) as well as non-endocrine cells (macrophages, endothelial cells, dendritic cells and fibroblasts).<sup>3</sup> The presence of such a variety of cellular components renders the investigation of causes leading to  $\beta$  cell destruction extremely difficult and, although studies on IDDM started decades ago,<sup>4</sup> the exact pathway(s) from insulinitis to the end-stage disease still remains unknown.<sup>5-7</sup>

Recent studies, however, have led to the formulation of hypotheses<sup>8,9</sup> giving clues on how the disease starts, develops and progresses. One major finding is the ability of non-endocrine cells, mainly activated macrophages, natural killer (NK) cells, as well as B and T lymphocytes, to produce diverse cytokines like interleukin-1 (IL-1 $\alpha$ , IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and interferon- $\gamma$  (IFN- $\gamma$ ).<sup>11-13</sup> These cytokines have been shown to induce  $\beta$  cells to undergo a number of

events like production of NO<sup>14,15</sup> and elevation of class II major histocompatibility complex (MHC) antigens.<sup>15-17</sup> They also interact with each other without excluding their potential synergist tendency with transforming growth factor-1 $\beta$  (TGF-1 $\beta$ ).<sup>18</sup>

The present work aims at the cellular events leading to  $\beta$  cell damage by IL-1, the possible delineation of the pathway(s) followed by this mediator and the possible reversibility of the destructive action which may be of value in the future. By thus employing the rat  $\beta$ -insulinoma cell system of RIN-5AH<sup>19</sup> as a model for IDDM, since these cells show *in vitro* what can happen to normal  $\beta$  cells *in vivo*, it is shown by apoptotic studies (BrdU labeling and Fas expression), intermediate product assessment (NO, PKC) and electron microscopy (SEM, TEM) that the degree of cell damage detected is neither the same nor similarly regulated by the two cytokines. However, in the case of IL-1 $\alpha$ , it can be ultimately reversed.

Therefore, it appears that IL-1-induced apoptosis of  $\beta$ -cells is a multi-stage process dependent upon a number of parameters, and that each isoform follows differently regulated pathways of action.

## Materials and Methods

### Cells and culture

The  $\beta$  cell-like line RIN-5AH used in this study was provided by Dr Herbert Oie.<sup>19</sup> Cells were grown in plastic culture flasks (Flow) in DMEM (Gibco) supplemented with 10% fetal bovine serum (FBS; Gibco) and maintained at 37°C in 5% CO<sub>2</sub> atmosphere.

### IL-1 reagents and induction protocol

Human recombinant IL-1 $\alpha$  and - $\beta$  were generous gifts from Dr Steven Gillis of Immunex Corp. (Seattle, WA), and used at concentrations of 12.5 and 25 ng/ml of culture, respectively. IL-1 $\alpha$  and - $\beta$  preparations from NCI (Mc Kesson BioServices, Rockville, MD) were also used at the same protein concentrations and gave similar results. In all but the BrdU labeling experiments, the cells were incubated with the above-stated amounts of IL-1 for 48 h. For the BrdU labeling, IL-1 exposure was for 2–8 h only in order to determine the presence of early apoptotic signals. In experiments where inhibitors were involved (inhibition of NO release), the iNOS inhibitor aminoguanidine (AG; Sigma) was added in the culture 18 h prior to IL-1 exposure.

### Other reagents

The Fas affinity-purified rabbit polyclonal antibody was purchased from Santa Cruz (CA) and used for surface labeling at a concentration of 1  $\mu$ g/ml. The BrdU labeling kit for DNA fragmentation assessment was purchased from Boehringer Mannheim (Germany) and used according to the manufacturer's instructions (see below). Antibodies to different PKC isoforms were purchased from Transduction Laboratories (Lexington, KY) and used at the suggested concentration by the supplier (1  $\mu$ g/test). Also from the same source were the specific antibodies to inducible- and endothelial-NOS (i- and e-NOS) similarly used at 1  $\mu$ g/test. Aminoguanidine (AG), a NOS inhibitor, was bought from Sigma (St Louis, MO) and used at 0.5 mM. Finally, all reagents for NO measurement (sulfanilamide and naphthylethylenediamine dihydrochloride) were also purchased from Sigma.

### NO measurement

Accumulation of NO in the medium of the IL-1-treated RIN cells was estimated by the determination of the concentration of the stable oxidation end-product nitrite by an automated colorimetric assay based on the Griess reaction as previously described.<sup>15</sup> Nitrite concentrations were calculated by comparison to sodium nitrite standards.

### Immunofluorescence

IL-1-induced RIN cells were processed for surface Fas expression as previously described,<sup>15</sup> whereas cytoplasmic PKC as well as i- and e-NOS detection was performed by first permeabilizing the cells and then proceeding as in the case of surface staining.<sup>20</sup>

### BrdU labeling

This assay has been applied to measure apoptosis by detection of BrdU-labeled DNA fragments in the cytoplasm of affected cells and/or necrosis by determining the amount of the same BrdU-labeled DNA fragments that have been released from the damaged target cells into the culture supernatants. It is based on the quantitative sandwich enzyme ELISA principle using two monoclonal antibodies against DNA and BrdU and was done using Boehringer's # 1 585 045 kit for cellular DNA fragmentation. Briefly, anti-DNA is fixed in 96-well plates where  $2 \times 10^3$  cells/well are added for the culture conditions already mentioned. After BrdU addition, the BrdU-labeled DNA fragments found in the test samples bind to the immobilized anti-DNA antibody and then the immunocomplexed fragments are denatured and fixed on the surface of the wells by microwave irradiation. Finally, anti-BrdU peroxidase conjugate reacts with the BrdU incorporated into the DNA and, after removing the unbound peroxidase conjugates, the amount of the complexed peroxidase is determined photometrically at 370–492 nm using 3,3',5,5'-Tetramethylbenzidine (TMB) as a substrate.

### Scanning electron microscopy (SEM)

For the SEM experiments, RIN-5AH cells were placed in 24-well plates (Sarstedt, Newton, NC) and cultured as described above with all the possible combinations of IL-1 and/or AG. In order to avoid cell damage by scraping, each well contained, at its bottom, a 13-mm diameter tissue culture coverslip (Sarstedt) which was simply removed for further processing. Thus, the adherent cells on the coverslip were fixed in 1% paraformaldehyde and 1% glutaraldehyde in 0.14 M sodium cacodylate buffer for 3 h. To preserve plasma membrane integrity, the cells were postfixed in 1% osmium tetroxide (OsO<sub>4</sub>) containing 0.1% ruthenium red in 0.14 M cacodylate buffer for 1 h at 4°C. All samples were dehydrated through an ascending ethanol series, critical point dried and coated with approximately 2 nm of platinum. The samples were examined in a JEOL (Tokyo, Japan) SEM at various magnifications and scanned accordingly.

### Transmission electron microscopy (TEM)

For TEM analysis, RIN-5AH cells were fixed in 2.5% glutaraldehyde in 5% sucrose in 0.2 M cacodylate

buffer for approximately 3 h at room temperature. Then the samples were postfixed in 1% OsO<sub>4</sub>, 5% sucrose and 0.1% ruthenium red for 2 h at 4°C. After a complete dehydration through a graded series of alcohol solutions, the cell pellets were embedded in L.R. White resin. Embedded samples were sectioned with an LKB Ultratome-V. Sections of 50–60 μm thickness were collected on 300-mesh nickel grids, quickly washed in PBS, and then washed in 1% BSA in PBS for 10 min. Images were obtained on Kodak electron microscope film 4489, using the JEOL-100C electron microscope, operated at 80 kV.

### Statistical analysis

In order to evaluate the statistical significance of the values between test samples and controls, all experiments were performed at least four times in triplicate, and the Student's *t*-test was employed for the assessment of significance levels (*P*).

## Results

### Demonstration of cell death by DNA fragmentation (BrdU incorporation)

In order to investigate cell death due to IL-1 exposure, BrdU incorporation was assessed 48 h after IL-1 addition to RIN cells. The results showed (Fig. 1A) that after 2 days the cells presented a necrotic state. However, this action, although statistically significant

(*P*<0.02), was not convincing since after 48 h of culture many events could have taken place and necrosis could have been the final event, thus missing possible earlier stages of apoptosis. Therefore, in order to exactly pinpoint the effect of IL-1α and IL-1β on these cells, we chose to repeat the experiments at earlier culture stages, that is at 2, 4, 6 and 8 h of IL-1 exposure. This approach permitted to demonstrate that both IL-1α and IL-1β initiated an early apoptotic signal, as detected by cytoplasmic DNA fragmentation, that was maximal 4 h after cytokine addition (Fig. 1B,C). When both interleukins were added to the cells simultaneously, a lower in magnitude apoptotic signal was seen at an even earlier time (2 h) which, however, was lost beyond 4 h of exposure (Fig. 1D). Thus, IL-1 appears to deliver early apoptotic signals to the β-insulinoma cells that will eventually lead the cells to their death. Thus, the necrotic signal obtained from the fragmented DNA in the culture supernatants at 48 h of IL-1 addition to the cells (as shown above; Fig. 1A) appears to be a normal consequence.

### Demonstration of cell death by assessing Fas expression and its correlation to NO production

Expression of Fas has been defined as an apoptotic marker<sup>8</sup>, whereas cells expressing Fas-L have been reported to promote cell death/apoptosis of Fas-bearing target cells when both populations come into contact.<sup>8,9</sup> Regardless of the constitutive or induced expression of Fas and/or Fas-L, the above situation applies *in vivo* where diverse cellular populations are present and interacting with each other. *In vitro*, however, the homogeneous cell populations used do not provide the matrix for cellular interactions that will facilitate or reproduce the *in vivo* situation. Thus, determination of the presence or absence of Fas is crucial since it may give clues as to whether presence or induction of this marker may account, by extrapolation, for cellular death in the living organism. Therefore, it was investigated whether IL-1 could induce Fas expression on the rat RIN cell line as it does with human and murine β cells.<sup>9,10</sup> After 48 h of culture, both IL-1α and IL-1β could induce significant levels of surface Fas expression over control (*P*<0.001 in both cases) suggesting its participation in apoptotic interactions (Fig. 2).

Cell death in IDDM has been linked to other mechanisms as well, one of which is production of NO (reviewed in Refs. 10 and 21). IL-1α and IL-1β do induce significant amounts of NO<sup>15,22,23</sup> which can be ablated by the NOS inhibitor aminoguanidine (AG),<sup>24</sup> that was found in our laboratory to be more potent than other inhibitors tested (L-NAME and L-NMMA;<sup>25</sup> data not shown). It thus became obvious to ask whether Fas and NO are related in their final task of cell death. For this, using the same NOS inhibitor, AG,

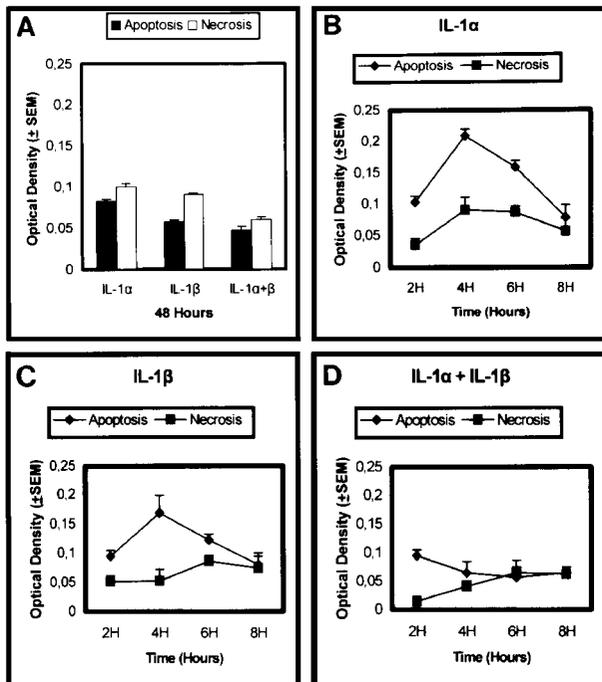


FIG. 1. Apoptosis vs necrosis on RIN-5AH cells after IL-1 exposure. (A) IL-1α and IL-1β after a 48-h treatment; (B) IL-1α at earlier times; (C) IL-1β at 2, 4, 6 and 8 h; (D) Combination of IL-1 isoforms.

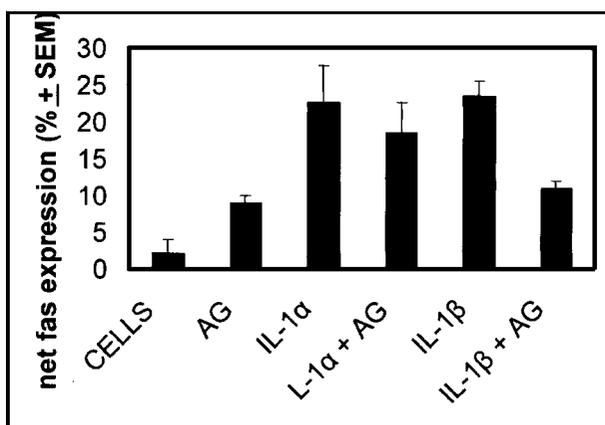


FIG. 2. Ability of IL-1 $\alpha$  and IL-1 $\beta$  to induce Fas expression and reversibility by the NOS inhibitor aminoguanidine (AG).

Fas expression was reassessed. It was found that, in the case of IL-1 $\alpha$ , AG was unable to inverse the induction of Fas, whereas in the case of IL-1 $\beta$  the inhibition was statistically significant ( $P < 0.001$ ; Fig. 2), suggesting a link between these two intermediates in IL-1 $\beta$  but not IL-1 $\alpha$  induction.

#### Assessment of the differential pathway of NO induction in RIN-5AH cells after IL-1 exposure

In order to explain and further examine the causes of the above-stated difference in Fas inhibition by AG after IL-1 $\alpha$  and IL-1 $\beta$  induction, it was hypothesized that IL-1 $\alpha$  and IL-1 $\beta$  could regulate NO production by different means. To study this possibility, immunofluorescence experiments with specific i- and e-NOS antibodies were performed. The results in Table 1 show that, in the case of IL-1 $\alpha$ , only the inducible form of NOS is activated leading to NO production, whereas in the case of IL-1 $\beta$  both i- and e-NOS are triggered. This latter observation ascribes to IL-1 $\beta$  the ability to lead RIN cells towards endothelial cell differentiation, a property not at all improbable, since IL-1's differentiation-inducing capacity on different cellular systems has been well described.<sup>26</sup> Such differential activation of the NOS isoforms by IL-1 $\alpha$

**Table 1.** Differential induction of i- and e-NOS by IL-1 $\alpha$  and IL-1 $\beta$  on RIN-5AH cells

	Net <sup>a</sup> percent ( $\pm$ SEM) of:	
	Anti-i-NOS	Anti-e-NOS
IL-1 $\alpha$	13 $\pm$ 1	1 $\pm$ 1
IL-1 $\beta$	5 $\pm$ 1	7 $\pm$ 2

<sup>a</sup> As assessed by immunofluorescence and by subtracting the control (untreated) staining which for both i- and e-NOS was 2  $\pm$  1%. According to this negative control value, all percentages obtained (except the IL-1 $\alpha$ -induced e-NOS) were statistically significant.

and IL-1 $\beta$  (only i-NOS versus dual i- and e-NOS, respectively), however, may also account for the relationship and/or regulation between IL-1-induced NO and IL-1-induced Fas. It may explain why AG, although inhibiting IL-1 $\alpha$ - and IL-1 $\beta$ -induced NO, only inhibits the IL-1 $\beta$ -induced Fas expression and not that of the IL-1 $\alpha$  as shown in Fig. 2.

#### Demonstration of cell death by scanning electron microscopy

In order to assess the degree of cell damage as well as the reversibility of this effect, scanning electron microscopy was employed.

#### IL-1 $\alpha$

Using the same 48-h IL-1 induction protocols with or without aminoguanidine, it was seen that IL-1 $\alpha$  destroyed the cells by opening holes and creating deep cuts by a degree greater than 65 and approaching 80% (Fig. 3C). This effect was completely ablated by aminoguanidine (by 85%; Fig. 3D) returning the cells to their initial appearance (Fig. 3A,B).

Therefore, since aminoguanidine (1) moderately, but significantly, inhibits IL-1 $\alpha$ -induced NO ( $P < 0.02$ ; data not shown) and (2) does not block IL-1 $\alpha$ -induced Fas, it appears that Fas and NO are two independent events. Their individual and indisputable involvement, however, in cell destruction does not apparently follow the same pathway. The link and the major point from these results, in conjunction with the finding that aminoguanidine inhibits IL-1 $\alpha$ -induced cellular damage (as seen by SEM in Fig. 2D), is that only NO via i-NOS (Table 1) and not Fas seems to actively participate in IL-1 $\alpha$ -induced apoptosis. As a note of caution, however, the role of IL-1 $\alpha$ -induced Fas cannot be underestimated since, as an apoptotic candidate molecule, it must be always considered as a causative agent acting in as yet undetermined ways (see Discussion for the possible interplay between Fas and protein kinase C).

#### IL-1 $\beta$

IL-1 $\beta$  gave different results, at least optically. Exposure of RIN cells to IL-1 $\beta$  revealed more swollen and hairy-like cells (by 70%) that had no apparent surface damage (Fig. 3E). This swollen type that results from the swelling of organelles, however, has been described as evidence of apoptosis,<sup>27</sup> which will eventually lead to cell rupture. Thus, since we obtain apoptotic signals by DNA fragmentation (Fig. 1C), and apoptotic nuclei by transmission electron microscopy (TEM; see below), it seems that indeed this unusually swollen appearance is indicative of apoptosis. Aminoguanidine could not alter anything of the IL-1 $\beta$  action morphologically (Fig. 3F).

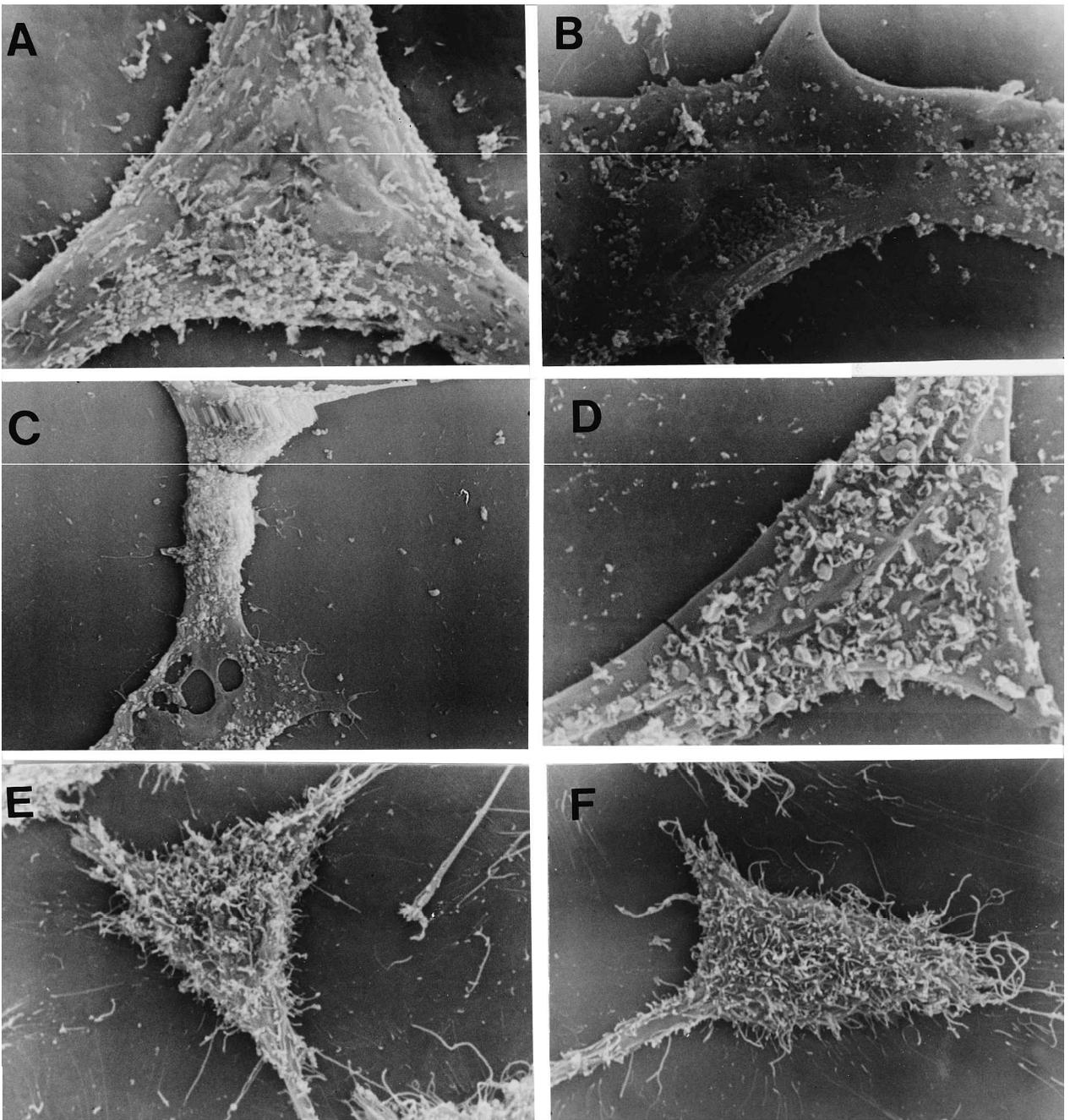


FIG. 3. Scanning electron microscopy of RIN-5AH cells treated with: (A) PBS at a magnification of  $\times 3300$ ; (B) aminoguanidine at  $\times 3500$ ; (C) IL-1 $\alpha$  at  $\times 2000$ ; (D) IL-1 $\alpha$ +aminoguanidine at  $\times 2500$ ; (E) IL-1 $\beta$  at  $\times 2000$ ; (F) IL-1 $\beta$ +aminoguanidine at  $\times 2000$ .

Therefore, since AG totally inhibits IL-1 $\beta$ -induced NO ( $P < 0.001$ ; data not shown) and IL-1 $\beta$ -induced Fas (Fig. 2), it can be claimed that Fas and NO are probably two linked events when induced by this IL-1 isoform, and that their relationship is found, as derived from Table 1, perhaps at the level of e-NOS regulation. However, since AG does not influence IL-1 $\beta$  cellular morphology, it can be cautiously claimed that neither Fas nor NO participate in the apoptotic event initiated by IL-1 $\beta$ , but they follow other

pathways in order to exercise their known destructive role on the RIN pancreatic population.

The combination of both IL-1 preparations gave mixed results (data not shown), where the separate effects of each IL-1 were seen combined at a degree greater than 70%. The addition of AG did not restore the cellular damage, a rather expected result if one considers the multiplicity of the possibilities as described above for each IL-1 isoform separately.

## Demonstration of cell death by transmission electron microscopy

For further ascertaining apoptosis, transmission electron microscopy was employed. Using the same experimental protocol of induction with IL-1 and the exact same timing as in the case of the scanning experiments (see above), the nuclei of the RIN cells were observed. The aim was to ascertain whether IL-1 could cause chromatin condensation, an indication of the apoptotic nucleus.<sup>28-30</sup> It was seen that, although aminoguanidine alone (not shown) did not affect the nuclear appearance, both IL-1 preparations caused some degree of chromatin condensation. This effect is marked by the presence of heterochromatin, which is transcriptionally inactive DNA, that tends to pack at the inner surface of the nuclear membrane. This situation could be reversed by AG only in the case of IL-1 $\alpha$  (approximately 80-85%) and not in the case of IL-1 $\beta$ . Representative images (Fig. 4A,B) show the combination of IL-1 $\alpha$  with AG (without chromatin condensation; reversal of effect) and IL-1 $\beta$ +AG (with presence of condensed chromatin).

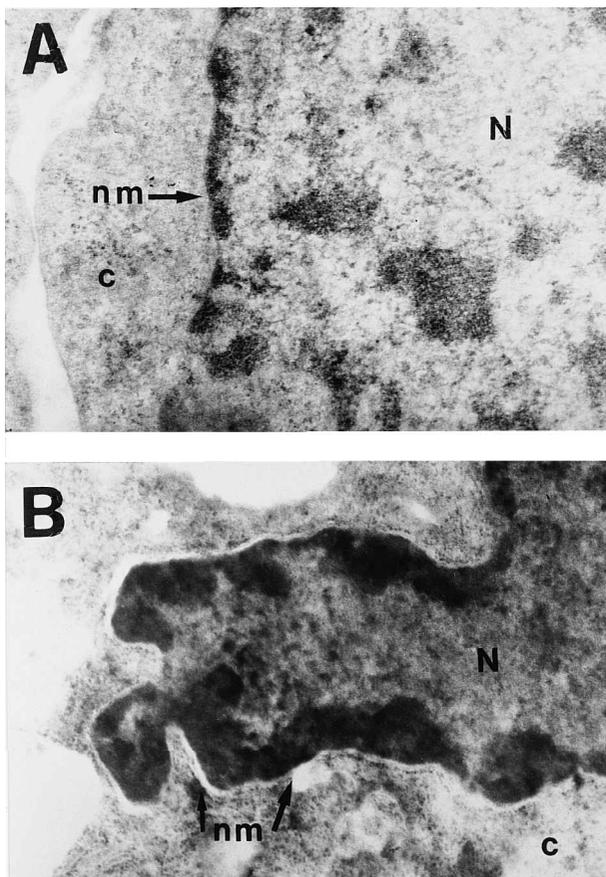


FIG. 4. Transmission electron microscopy of RIN-5AH cells treated with: (A) IL-1 $\alpha$ +AG where no condensed chromatin is obtained;  $\times 20\,000$ ; (B) IL-1 $\beta$ +AG with condensed nuclear chromatin;  $\times 13\,000$  (N, nucleus; nm, nuclear membrane; c, cytoplasm).

## Discussion

### IL-1-induced apoptosis in IDDM

This work has shown that IL-1 induces early apoptotic signals to the  $\beta$ -insulinoma RIN-5AH cells that have been used as a model of IDDM, since the study of those cells *in vitro* is indicative of what happens to normal  $\beta$  cells *in vivo*. This early action is accompanied by a number of other events, some of which have also been studied here. These include DNA fragmentation, NO production and Fas up-regulation.

Depending on the IL-1 isoform, it can be seen that the way the intermediates behave towards cell destruction differs enormously:

First, in IL-1 $\alpha$ -induced apoptosis, NO is shown to actively participate/synergize in the final task of cell death, whereas the role of Fas seems, although documented and widely accepted,<sup>9,10</sup> independent of IL-1 $\alpha$ . An important point of great interest and with future implications is the reversibility of cell damage by aminoguanidine.

Second, concerning IL-1 $\beta$ -induced apoptosis, although NO and Fas seem to proceed together to their destructive role, since they appear to be two linked events, neither convincingly synergizes with IL-1 $\beta$ .

This differential behavior in apoptosis in terms of cellular appearance and regulation of intermediates followed by the two IL-1 isoforms was not, however, unexpected. Our laboratory has shown that IL-1 $\alpha$  and IL-1 $\beta$  binding on specific and, at the same time, distinct surface receptors on these very same cells follows different kinetics.<sup>15</sup> In addition, the signals transduced via these receptors for the induction of surface class II histocompatibility antigens, and probably for other regulatory molecules, have also been demonstrated to be different.<sup>15,20</sup>

The majority of the results presented here on apoptosis are in agreement with the works of many laboratories, as presented in a recent review article by Mauricio and Mandrup-Poulsen.<sup>27</sup> However, it must be noted that most of the work done on IDDM concerns the actions of IL-1 $\beta$  and not that of IL-1 $\alpha$ . This is probably due to the controversial results usually obtained with IL-1 $\alpha$ . This fact can be explained by the presence of not uniformly and continuously up-regulated IL-1 $\alpha$  receptors<sup>15</sup> on  $\beta$  cells that, according to their density at the time of the experiments, seem capable of transducing different signals, as mentioned already.

Is there any PKC involvement in IL-1-induced cellular damage?

According to a recent publication by Wang *et al.*,<sup>31</sup> it is suggested that in a variety of cellular systems PKC regulates Fas expression. In addition, we have

previously published that IL-1 $\alpha$  induces the expression of PKC on the RIN-5AH cells by a significant net percentage of 30%, as assessed by a pan-PKC monoclonal antibody (Upstate Biotechnology, Lake Placid, NY). On the contrary, IL-1 $\beta$  can only trigger the cells to express a non-significant net 5% (5+1% in the untreated control, 35+6% with IL-1 $\alpha$  and 10+1% with IL-1 $\beta$ ).<sup>20</sup>

From the present work it can be seen that IL-1 $\alpha$  induces a significant amount of Fas (Fig. 2), while it is also able to induce a significant amount of total PKC.<sup>20</sup> On the other hand, IL-1 $\beta$ , which triggers the cells to marginally express total PKC,<sup>20</sup> induces the same levels of Fas compared to IL-1 $\alpha$  (Fig. 2). Although these results are not exactly compatible, unpublished data from our laboratory have shown that the constitutive PKC $\alpha$  expression of RIN cells can be blocked by IL-1 $\beta$  (from 22 to 4%;  $P < 0.001$ ), while PKC $\beta$  is augmented from 0 to 8% ( $P < 0.02$ ), the  $\mu$  isoform is marginally increased from 0 to 3% ( $P < 0.25$ ), PKC $\zeta$  remains unaffected (4–6%) and the  $\epsilon$  isoform decreases from 4 to 0% ( $P < 0.25$ ). While all other PKC isoforms are not expressed on untreated RIN cells, and not modulated by at least IL-1 $\beta$ , it is possible that there exists an intracellular cooperation between Fas and total or isoform-specific PKC, in conjunction with cellular damage initiated by IL-1. Such a possibility can be examined by the use of PKC inhibitors (i.e. sphingosine at 50  $\mu\text{M}$ <sup>20</sup>), since this approach may give answers to these and other relevant questions, currently investigated by our laboratory.

Thus, from the present study, it is apparent that IL-1 $\alpha$  and IL-1 $\beta$  follow their own regulatory pathway(s) concerning  $\beta$ -cell damage. Regardless, however, of the IL-1 isoform or any other cytokine/mediator employed, the study of  $\beta$ -cell destruction in IDDM remains a difficult task to pursue, since the number of intermediates involved indicate the complexity of the cellular interactions leading to cell death.

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