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Activation of peritoneal macrophages during the prediabetic phase in low-dose streptozotocin-treated mice

Josefa Andrade, Manuel Conde, Francisco Sobrino and Francisco J. Bedoya

Laboratorio de Bioquímica de Sistemas Inmunológicos, Departamento de Bioquímica Médica y Biología Molecular, Avda. Sánchez Pizjuán, 4, 41009 Sevilla, Spain

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Glucose metabolism and the production of O_2^- and NO_2^- have been studied in peritoneal macrophages from mice injected with 5 subdiabetogenic doses of streptozotocin. On day 12 after beginning of the treatment, peritoneal macrophages produced significantly higher amounts of lactate than macrophages from control mice. In addition, NO_2^- release and phorbol ester-induced O_2^- production were significantly augmented in macrophages from streptozotocin-treated mice. γ-Interferon induced in a dose-dependent manner the activity of NO synthase only in macrophages from streptozotocin-treated mice These data show for the first time that peritoneal macrophages from streptozotocin-treated mice are activated and produce effector molecules such as O_2^- and 'NO which could participate in the destruction of pancreatic islets.

Nitric oxide, Superoxide anion, Diabetes; Macrophage; γ-Interferon

1. INTRODUCTION

Macrophages play a pivotal role in the immune attack against the pancreatic islets in several animal models of type I diabetes [1,2]. Recently, it has been shown that islet cell lysis induced by *C. parvum*-activated macrophages is mediated by 'NO since cell destruction can be counteracted by NO synthase inhibitors [3]. However, evidence concerning the activation state of macrophages and the effector molecules that mediate islet destruction in experimental immune diabetes is lacking. By studying the production of lactate and the release of effector molecules such as O_2^- and 'NO, we show for the first time that peritoneal macrophages from low-dose streptozotocin-treated mice are activated at a time when the immune destruction of the pancreatic islets is in progress.

2. MATERIALS AND METHODS

Male CD-1 mice (Charles River, Barcelona, Spain) weighing 25–30 g were used. Culture media were from ICN (Barcelona, Spain) FCS, antibiotics, streptozotocin (STZ), cytochrome *c*, Phorbol 12-myristate 13-acetate (PMA), lactate dehydrogenase and NAD^+ were from Sigma (Alcobendas, Spain). Salts were from Merck (Barcelona, Spain). γ-Interferon (IFN-γ) and superoxide dismutase were from Boehringer Mannheim (Barcelona, Spain).

Correspondence address F.J. Bedoya, Laboratorio de Bioquímica de Sistemas Inmunológicos, Depto. de bioquímica Médica y Biología Molecular, Adva. Sánchez Pizjuán, 4, 41009 Sevilla, Spain. Fax: (34) (5) 490 7041.

2.1 *STZ administration*

STZ was dissolved in cold citrate buffer, pH 4.5, and injected within 3 min i.p. at a dose of 40 mg/kg on 5 consecutive days

2.2. *Harvest of macrophages*

Peritoneal macrophages were harvested by peritoneal lavage [4]. The viability of the collected cells was estimated from their ability to exclude Trypan blue and was always higher than 95%.

2.3. *Cell culture*

Peritoneal cells were resuspended in culture medium consisting of either RPMI 1640 or EMEM without arginine, 10% FCS and antibiotics and plated (8×10^5 cells in 250 μl) in flat bottom, 96-well culture plates and incubated for 48 h at 37°C

2.4 *Assay of superoxide anion production*

O_2^- production was measured as in [5]. Briefly, macrophages (10^6 cells/ml) were incubated at 37°C in Krebs-Ringer bicarbonate buffer containing 10 mM glucose and 80 μM cytochrome *c*. After 5 min, 100 nM PMA was added and superoxide dismutase-inhibitable decrease in absorbance at 550 nm was recorded contiuously. The rate of O_2^- generation was estimated using a molar absorption coefficient of 21.1 $mM^{-1} \cdot cm^{-1}$

2.5 *Lactate determination*

Lactate accumulation in medium was measured using a standard spectrophotometric assay [6]

2.6. *Nitrite determination*

The accumulation of NO_2^- in culture supernatants was measured with the Griess reagent [7]. Briefly, 100–150 μl of supernatant was mixed with 500 μl of Griess reagent. Absorbance was measured at 550 nm NO_2^- concentration was calculated from a $NaNO_2$ standard curve.

3. RESULTS

Fig. 1 shows that the treatment with low-dose STZ induced an increase in the PMA-induced O_2^- production

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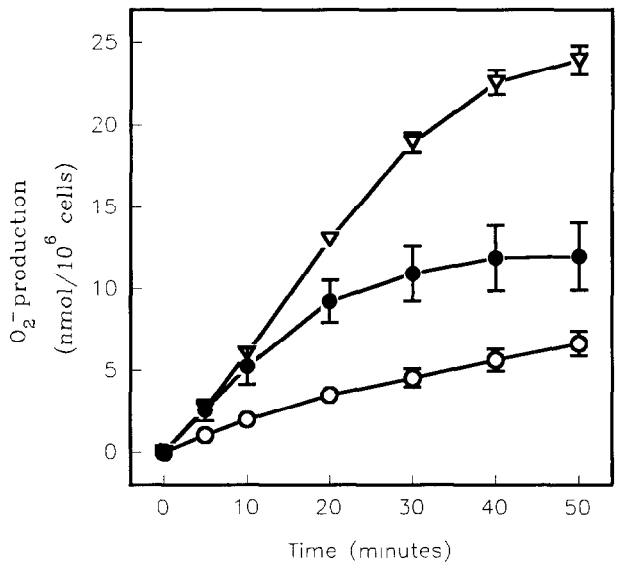

Fig. 1. Evolution of superoxide anion production by peritoneal macrophages from mice treated with low-dose STZ. Superoxide anion production was measured at day 6 (●—●) and at day 12 (▽--▽). Control mice (○—○) received buffer alone. Values are means ± S.E.M. for three experiments performed in triplicate.

by peritoneal macrophages. On day 6 after beginning of the treatment with STZ, O_2^- production was increased 79% over control. This increase was more evident when the time elapsed was 12 days (259% over control).

Table I shows that NO_2^- production is significantly increased in macrophages from the 12-day group. Lactate production by macrophages was also significantly higher than macrophages from control mice.

Fig. 2 shows the IFN-γ dependence of NO_2^- production by macrophages cultured in EMEM medium without arginine. Under these conditions, basal NO_2^- production by STZ-treated macrophages is significantly lower than control macrophages. IFN-γ selectively stimulates NO_2^- production by cultured STZ-treated macrophages in a concentration-dependent manner, since no effect of IFN-γ was detected in control macrophages.

Table I

Nitrite production and lactate output in macrophages from control and STZ injected CD-1 mice

	NO_2^- (nmol/well)	Lactate (μmol/well)
Controls	1.86 ± 0.15	0.32 ± 0.05
STZ-treated	9.29 ± 2.32*	0.90 ± 0.12*

Macrophages (5×10^5 cells per well were collected from citrate-injected mice and from mice 12 days after the first STZ injection and cultured in RPMI 1640 for 48 h. Values are the means ± S.E.M. from 5 replicates and are representative of 4 separate experiments. *$2P < 0.002$.

Table II shows the effect of IFN-γ on NO_2^- production by macrophages from *E. coli*-treated mice. Following culture in EMEM medium without arginine for 48 h, *E. coli*-activated macrophages produce significantly higher amounts of NO_2^- than control macrophages. In addition, IFN-γ (100 U/ml) specifically stimulated the production of NO_2^- in *E. coli*-activated macrophages.

4. DISCUSSION

The administration of multiple low doses of STZ to CD-1 mice results in a progressive destruction of pancreatic β-cells [8]. Macrophages have been reported to be the cells that initiate the immune attack against the pancreatic β-cells in this experimental model [1]. We have found that peritoneal macrophages from STZ-treated mice display an enhanced production of O_2^- in response to PMA (Fig. 1). This increase in O_2^- generation by macrophages is a consequence of their activation and may affect pancreatic β-cells since superoxide dismutase activity is low in these cells [9,10].

Peritoneal macrophages collected 12 days after beginning of the STZ treatment produced significantly higher amounts of lactate than macrophages from control mice (Table I). Similar findings have been reported in *E. coli*- and in *C. parvum*-activated macrophages and indicate that these cells metabolize glucose at high rate to supply energy for the activation processes [6,11]. 'NO production (measured as nitrite accumulation in the culture medium) is also increased after STZ treatment at satu-

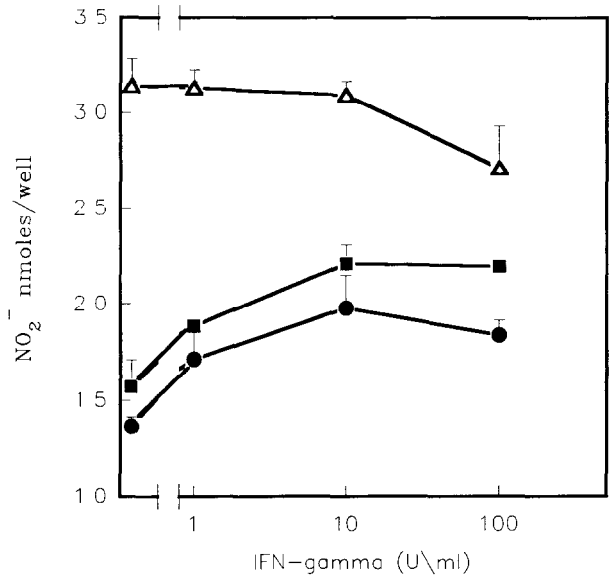

Fig. 2. Effect of γ-interferon on NO_2^- production by peritoneal macrophages. Macrophages (8×10^5 cells per well) were incubated for 48 h with different amounts of IFN-γ in EMEM medium without arginine. Macrophages were from control mice (▽—▽), from STZ-injected mice at day 12 (■—■) and from STZ-injected mice at day 21 (●—●) Data are means ± S.E M. from triplicates and are representative of 2–3 experiments.

Table II

Effect of IFN-γ on NO_2^- production by *E. coli*-activated macrophages

	NO_2^- (nmol/well)	
	Without IFN-γ	+100 U/ml IFN-γ
Controls	2.28 ± 0.03	2.29 ± 0.06*
E coli-activated	6.09 ± 0.20	10.17 ± 0.39**

Macrophages (5×10^5 cells per well) from control mice and from *E coli*-treated mice were cultured in an arginine free medium for 48 h in the presence of 100 U/ml IFN-γ. Values are means ± S.E M from triplicates and are representative of 2 experiments.
*NS, **$2P < 0.001$.

rating concentrations of arginine (Table I). These results show that NO is released by macrophages at a time when macrophage infiltration of the islet is in progress [1]. It is thus possible that the generation of 'NO by activated macrophages is critical for the destruction of the β-cell in this model of immune mediated diabetes as is the case in *C. parvum*-activated macrophages [3].

IFN-γ is a potent inducer of 'NO synthase by macrophages from several strains of mice and rats and may play a regulatory role on the activation of macrophages during the immune attack to the β-cells [12,13]. We have found that IFN-γ stimulates selectively NO_2^- production by macrophages from *E. coli*-treated mice and by macrophages from STZ-treated CD-1 mice (Table II and Fig. 2). It is thus entirely possible that IFN-γ released by helper lymphocytes located in the vicinity of pancreatic β-cells may modulate macrophage mediated islet cell destruction.

In conclusion, our data show that peritoneal macrophages are metabolically active and have an increased production of O_2^- and 'NO at a time when immune attack to the endocrine pancreas is in progress. The actions of macrophages may be controlled by cytokines such as IFN-γ released by lymphocytes in the vicinity of the β-cells in this experimental model of type I diabetes.

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