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Tumour necrosis factor alpha production is upregulated in diabetes prone BB rats

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Summary. Following activation peritoneal macrophages from diabetes prone BB rats secreted strikingly higher amounts of tumour necrosis factor alpha than found for macrophages from diabetes resistant or normal Wistar rats. Enhanced tumour necrosis factor alpha production was detected prior to the occurrence of insulitis. Cultures of macrophages derived from precursor cells in diabetes prone BB rat bone marrow also showed upregulated tumour necrosis factor alpha secretion upon challenge with endotoxin and interferon gamma. Tumour necrosis factor alpha hypersecretion may contribute to autoimmune diabetes by affecting thymic and post-thymic T-cell maturation and by promoting pancreatic islet inflammation.

Key words: BB rat, autoimmunity, tumour necrosis factor alpha, macrophages.

The development of Type 1 (insulin-dependent) diabetes mellitus in humans as well as in most of its animal models is T-cell dependent [1] but a significant contribution of macrophages to disease development was noted. Macrophage directed immune intervention prevents diabetes development, macrophages precede T cells in islet inflammation [1], macrophages lyse islet cells in vitro [2] and macrophage products interleukin 1 (IL-1) and tumour necrosis factor α (TNFα) interfere with islet Beta-cell function and viability [3, 4].

This particular role of macrophages in diabetes pathogenesis prompted us to search for defects of macrophage function in BB rats. We have analysed the production by macrophages of TNFα, a major immunoregulatory, proinflammatory and cytotoxic cytokine.

Materials and methods

Rats and macrophage isolation

Peritoneal macrophages of diabetes-prone BB/W Dus rats, diabetes resistant BB/WD rats (both 65–70 days of age) and Wistar Wi/Han rats (65–90 days of age, kindly provided by Dipl. Biol. U. Kiesel, Düsseldorf, FRG) were activated in vivo by i. p. injection of 500 µl of heat inactivated *Corynebacterium parvum* (Wellcome, Burgwedel, FRG). Peritoneal cells were isolated four days later by peritoneal lavage and macrophages were enriched by adherence for 2 h (37 °C, 5% CO_2) in RPMI 1640 with 10% fetal calf serum (FCS) on plastic dishes coated with FCS. Adherent cells were detached by incubation with 3 ml ice-cold Ca^{2+}-, Mg^{2+}-free Hank's Balanced salt solution (Merck, Darmstadt, FRG) with 25 mmol/l EDTA for 7 min. Bone marrow cells were isolated by flushing femora of 58–66 days old animals followed by culture in RPMI 1640 plus 15% colony stimulating factor 1 (CSF-1) containing medium at a concentration of 4×10^6 cells/ml for 4 days and a further 24 h in RPMI 1640 + 10% FCS. Supernatants of cultures of L929 cells (2×10^5 cells/ml) were used as source of CSF-1.

Analysis of TNF production

Adherent peritoneal cells (1×10^6 cells/ml) were incubated over night in RPMI 1640 with 10% FCS and afterwards stimulated in vitro with 1 µg/ml lipopolysaccharide (LPS from *Escherichia coli* 0 26: B6, Sigma, Munich, FRG) and supernatant withdrawn after 0, 5 and 25 h. Bone marrow derived macrophages were stimulated with 1 µg/ml LPS or with 10 ng/ml LPS plus 100 U/ml recombinant rat interferon gamma (Holland Biotechnology bv, Leiden, Netherlands). TNFα was quantitated by a bioassay using L929 cells as described [5]. Control experiments were performed with a rabbit anti murine TNFα serum (not crossreacting with TNFβ, kind gift of Dr. G. R. Adolf, Boehringer-Institute, Vienna, Austria). Of the antiserum 116 nl were found to neutralise one unit of recombinant murine TNFα (Genzyme, Boston, Mass., USA). Characterization of peritoneal and bone marrow cells was done by immunochemistry as described previously [2] by using mouse anti-rat mAb: W3/25 specific for CD4 marker on a fraction of macrophages and T lymphocytes; W3/13 specific for lymphocytes and polymorphonuclear cells; 0 × 19 specific for T lymphocytes; 0 × 8 specific for CD8 marker on a fraction of T lymphocytes and Natural Killer cells (all from Camon-Serotec, Wiesbaden, FRG); ED1 specific for a cytoplasmic vacuole antigen on some free and tissue macrophages and ED2 specific for a

Table 1. Characterization of adherent peritoneal and bone marrow derived cells from diabetes prone, diabetes resistant BB and normal Wistar rats

Monoclonal antibody	Adherent peritoneal cells			Adherent bone marrow derived cells	
	Diabetes prone BB	Diabetes resistant BB	Wistar	Diabetes prone BB	Wistar
			(% positive cells)		
ED1	>95	>95	>95	91±1.5	93±1.5
ED2	4.0±0.5	4.5±0.4	3.9±0.6	8±1.7	6±1.4
W3/25	8.5±1.4	7.3±1.0	7.8±1.3	7±3.2	8±2.5
W3/13	6.0±0.9	6.5±1.0	5.9±1.2	8±2.7	8±3.2
Ox 19	2.0±1.1	2.5±0.8	2.2±1.0	5±2	6±1.8
Ox 8	<1	<1	<1	2±1	2±1
Asialo GM1	<1	<1	<1	1±1	1±1

Peritoneal macrophages were enriched by adherence for 2 h (37 °C, 5% CO_2) on plastic dishes coated with fetal calf serum. The adherent fraction contained a mean of 33×10^6 cells for diabetes prone BB ($n = 5$), 20×10^6 for diabetes resistant BB ($n = 7$) and 45×10^6 for Wistar rats ($n = 10$). Adherent bone marrow cells were analysed after culture for 4 + 1 days (see Materials and methods)

membrane antigen on some tissue macrophages (kind gift of Dr. C.D. Dijkstra, Amsterdam, Netherlands); anti-asialo GM1 (Wako Chemicals GmbH, Neuss, FRG) specific for Natural Killer cells and some T cells.

Screening of pancreata for insulitis was done by preparation of haematoxylin-eosin stained sections of Bouin-fixed, paraffin embedded pancreata and reading of coded slides by two observers.

Results

Peritoneal macrophages were harvested from diabetes prone, diabetes resistant BB rats or normal Wistar rats after activation in vivo with *Corynebacterium parvum*. Comparable numbers of macrophages were isolated in the three strains and their purity and the expression of ED1 and ED2 markers were highly similar (Table 1). Macrophages were restimulated in vitro with lipopolysaccharide and the culture supernatant withdrawn at 0, 5, 7 or 25 h for determination of its TNFα content. As shown in Table 2 basal and maximal TNFα secretion was strikingly higher in macrophages from diabetes prone BB rats (basal: 24 U/10^6 cells; peak 1300 U/10^6 cells) than in macrophages from diabetes resistant BB rats (basal <0.5 U/10^6 cells). The latter results were similar as seen with macrophages from Wistar rats.

Table 2. Enhanced TNFα secretion by macrophages from diabetes prone BB rats

Peritoneal macrophages	n	Stimulation in vitro	TNFα secretion (U/10^6 cells ± SD) 0 h	5 h	25 h
Diabetes prone BB	5	LPS	24±2	1300±25	208±9
Diabetes resistant BB	7	LPS	<0.5	52± 4	4±1
Wistar	10	LPS	<0.5	36± 3	2±1
Bone marrow derived macrophages					
Diabetes prone BB	7	LPS	<0.5	136±39	<0.5
	4	LPS + IFNγ	<0.5	147± 5	<0.5
Wistar	7	LPS	<0.5	30± 3	<0.5
	4	LPS + IFNγ	<0.5	64±17	<0.5

Adherent bone marrow derived cells contained more than 90% of ED1$^+$ macrophages for both diabetes prone BB and Wistar rats (Table 1). These cells were activated in vitro with LPS or a combination of LPS and IFNγ. In both cases macrophages from diabetes prone BB rats secreted higher amounts of TNFα than were found for cells from Wistar rats. Diabetes resistant BB rats were not available for this study. Control experiments were performed to verify that the cytokine measured by the L929 bioassay was TNFα and not a related cytokine such as TNFβ. Macrophage culture supernatants from the three rat strains were mixed with 1/100 volume of the TNFα antiserum or with rabbit normal serum. Lysis of L929 cells was completely blocked in the presence of the TNFα antiserum (less than 5% lysis above background levels in each instance).

Of the diabetes prone BB rats a total of 106 islets were analysed for signs of islet inflammation. We found two islet sections >10 mononuclear cells infiltrated and one islet section showed a small cluster of five infiltrating cells. All the other islet sections were devoid of recognizable pathological changes.

Discussion

After i.p. injection of *C. parvum* activated macrophages of similar number and phenotype could be isolated from normal Wistar rats and from age- and sex-matched diabetes prone or diabetes resistant BB rats. Even so, striking differences were found in the levels of basal and LPS-induced TNFα-production. Macrophages from diabetes-resistant BB rats lacked demonstrable basal TNFα-production and responded moderately to the LPS-stimulus. Similar kinetics were seen in Wistar rats. In contrast, macrophages from diabetes prone BB rats showed higher basal TNFα-levels in the supernatant as well as enhanced peak concentrations. For convenience the enhanced TNFα-level in supernatants of activated macrophages is termed TNFα hypersecretion. However, it must be noted that decreased removal/degradation of secreted TNFα may also be involved. Preliminary experiments show enhanced TNFα mRNA levels (unpublished observations).

Upregulated TNFα production was observed in non-diabetic BB rats. In fact, at the time of peritoneal cell harvest BB rats showed only minimal signs of insulitis.

It is of interest that a difference in TNFα secretion between diabetes prone BB and Wistar rats was also found when macrophages derived from bone marrow cells by in vitro culture were tested. This finding indicates that the regulatory defect described is endogenous to this cell lineage. Studies are underway to determine whether TNFα hypersecretion is genetically linked with other immune cell abnormalities in the BB rat such as lymphopoenia and the defect in RT6* expression by T cells.

TNFα is a proinflammatory cytokine of which overproduction may favour chronic local inflammation such as pancreatic insulitis. In addition, TNFα in conjunction with interleukin 1 and interferon gamma shows direct toxic effects on islet cells in vitro [4]. Finally, TNFα is a co-stimulatory signal during antigen presentation. Upregulated TNFα production may interfere with proper thymic and post-thymic T-cell stimulation/maturation and thereby promote islet autoimmunity.

Abnormal cytokine release by macrophages has been described recently in several autoimmune diseases. In some cases cytokine secretion is enhanced [6], in others it is decreased [7]. In spontaneously diabetic NOD mice TNFα production is decreased and exogenous TNFα may even prevent diabetes development [8]. In human Type 1 diabetes HLA-DR types conferring protection seem to be associated with decreased TNFα secretion by monocytes, but a general consensus has not yet been reached [9, 10]. We therefore assume that aberrant TNFα production in general, be it increased or decreased, contributes to immune dysregulation thus favouring the development of autoimmunity.

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