

Mechanisms of Pancreatic Islet Cell Destruction

Dose-Dependent Cytotoxic Effect of Soluble Blood Mononuclear Cell Mediators on Isolated Islets of Langerhans

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Supernatants of peripheral blood mononuclear cells from healthy human donors stimulated with recall antigen (purified protein derivative of tuberculin) or lectin (phytohaemagglutinin) markedly inhibited the insulin release from isolated human and rat islets of Langerhans, and decreased rat islet contents of insulin and glucagon in a dose-dependent manner. A maximal effect on islet function was obtained with supernatant concentrations down to 5%. Supernatants of mononuclear cells stimulated with tuberculin were more potent than supernatants produced by lectin stimulation. Culture medium reconstituted with tuberculin or phytohaemagglutinin did not impair islet function. Electron microscopy demonstrated that supernatants were cytotoxic to islet cells. The cytotoxic mononuclear cell mediator(s) was non-dialysable, sensitive to heating to 56°C, labile even when stored at -70°C, but stable when lyophilised.

Key words: autoimmunity; cell-mediated immunity; cytotoxicity; cytokines; lymphokines; pancreatic islets; Type 1 (insulin-dependent) diabetes mellitus.

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CLINICAL ASPECTS

Activated mononuclear cells can produce antigen-non-specific cytotoxic cytokine(s). It is suggested that beta-cell destruction in Type 1 diabetes mellitus, the hallmark of which is mononuclear cell infiltration of the islets of Langerhans, may be caused by such cytokine(s).

There is abundant circumstantial evidence to suggest that Type 1 (insulin-dependent) diabetes mellitus is caused by an autoimmune attack on pancreatic beta-cells (19). The molecular mechanism underlying the autoimmune destruction of the beta-cells leading to Type 1 diabetes in man is not clear. A pathogenetic importance of circulating autoantibodies reacting with islet cell antigens has been suggested, although direct evidence is lacking (13, 19). Indeed, presence or absence of islet cell cytoplasmic antibodies was unrelated to the

higher than expected remission rate in cyclosporin-immunosuppressed newly diagnosed Type 1 diabetics (15). Experience with segmental pancreatic transplantation between discordant monozygotic twin pairs showed that beta-cells in the pancreatic grafts from the healthy twin donors were selectively destroyed by the diabetic recipients within 8 weeks of transplantation. Pancreatic biopsies demonstrated prominent mononuclear cell infiltration of primarily T-cells in the islets (insulinitis), no IgG deposits in the islets, no HLA-DR expression on islet

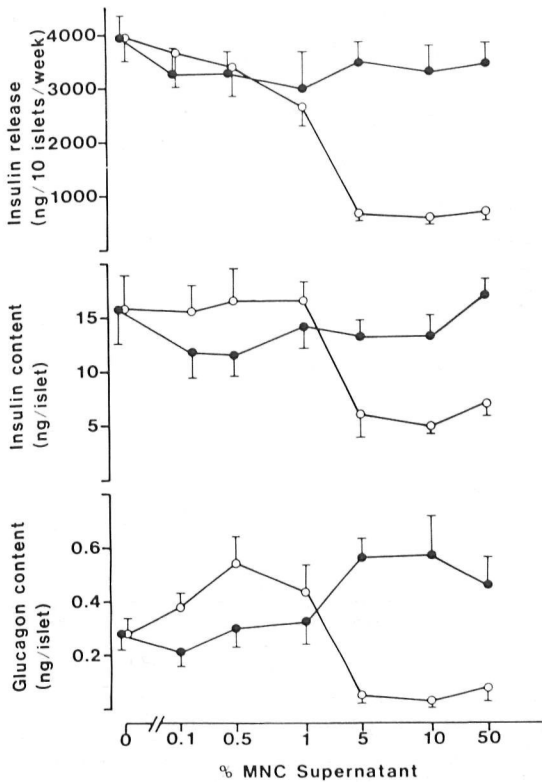


Fig. 1. Effects of 7 days of culture with varying concentrations of supernatants of unstimulated MNC (●) or MNC stimulated with PPD (○) on rat islet function. Each point is the mean of duplicate experiments with four separate islet preparations and supernatants from MNC isolated from one donor.

Effect on accumulated glucose-induced insulin release to the culture medium (top panel), on islet content of insulin (middle panel) and glucagon (lower panel).

cells, and no vasculitis as a sign of graft rejection. Appearance of circulating islet auto-antibodies was not observed after transplantation (24). Recent onset Type 1 diabetics show delayed (type IV) hypersensitivity to islet antigen preparations (18), and T-cells from such patients inhibit insulin release from or are cytotoxic to xenogeneic islet cells *in vitro* (3, 5).

Antigen-non-specific soluble peptides (cytokines) produced by activated macrophages (monokines) and lymphocytes (lymphokines) are essential mediators in the cell-to-cell interaction in the immune network and in the cell-mediated immune response (1, 23). Many of these factors are hormone-like in action, i.e.

they are secreted in minute quantities and exert their effects by binding to specific surface receptors on various target cells. Cytokines have multiple functions in the interplay between monocytes and T- and B-lymphocytes, but only a few have been defined at the molecular level. The cytokines have therefore been classified primarily according to their biological activity, e.g. as T- and B-lymphocyte growth and differentiation factors, chemotactic factors, migration inhibitory factors, etc. (Table 1). It was not known whether cytokines secreted by mononuclear cells in the autoimmune infiltrate contribute to target cell destruction.

We have previously reported that crude cytokine preparations from normal human donors decreased the insulin release from isolated human and rat islets of Langerhans, and the content of insulin and glucagon in isolated rat islets (16). Light microscopy revealed that these effects were due to a cytotoxic effect of the cytokine preparations. The purpose of the present work was to study this in further detail to define the dose-response relationship of the effects, and to investigate some physico-chemical properties of the active component(s) in the cytokine preparations.

Table 1

Some important mediators of immunity (cytokines)

1) Monokines (products of activated monocytes and macrophages)
Interleukin 1 (IL-1, lymphocyte-activating factor, B-cell activating factor, monocyte pyrogen)
Colony-stimulating activity (CSA)
Proteinase (plasminogen activator, elastase, collagenase)
Complement components
2) Lymphokines (products of activated T-cells)
Macrophage activating factor (MAF)
Macrophage chemotactic factor
Leucocyte chemotactic factor
Leucocyte migration inhibitory factor (LIF)
Interleukin 2 (IL-2, T-cell growth factor)
B-cell growth factor (BCGF)
B-cell differentiation factor (BCDF)
Lymphotoxins
Interferons
Osteoclastic factor
Interleukin 3 (haematopoietic cell growth factor)

MATERIAL AND METHODS

Production of supernatants of blood mononuclear cells

Cell-free supernatants of unstimulated as well as stimulated peripheral blood mononuclear cells (MNC) from healthy blood donors were prepared as described (16). MNC were separated from dextran-sedimented venous blood by Ficoll gradient centrifugation. Control supernatants were obtained from 22-h cultures of 2.5×10^6 MNC/ml culture medium TC 199 (Gibco, Paisley, Scotland) supplemented with 25 mmol/l hepes (pH 7.4), 500,000 IU/l penicillin, 500 mg/l streptomycin and 1 % heat-inactivated human serum pool. Active supernatants were obtained from parallel cultures challenged by a) 50 mg/l purified protein derivative of tuberculin (PPD, State Serum Institute, Copenhagen, Denmark), or b) pulse-treatment for 1 h with 5 mg/l phytohaemagglutinin (PHA, PHA-P, Difco, Michigan, USA).

The supernatants were decanted after centrifugation at 400 g for 10 min and either tested immediate or dialysed against NH_4HCO_3 -buffer adjusted to pH 7.2 with acetic acid and lyophilised. The lyophilised material was stored at -20°C and dissolved in the original volume of serum-free medium RPMI 1640 (Flow Laboratories, Irvine, Scotland) supplemented with 20 mmol/l hepes (pH 7.4), 100,000 IU/l peni-

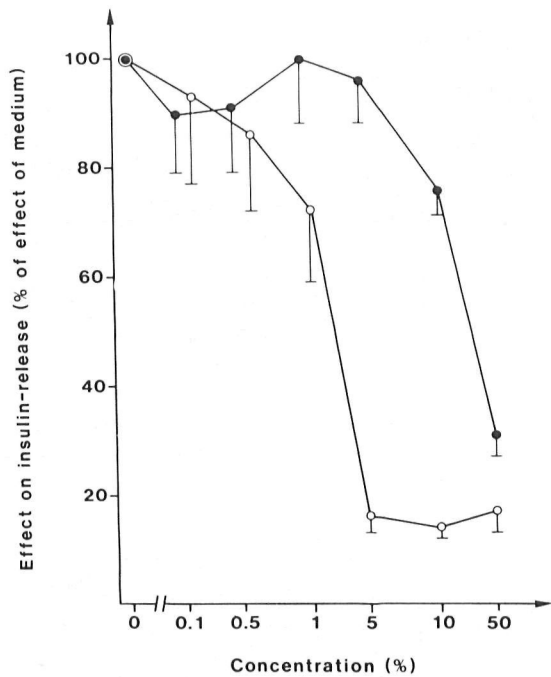


Fig. 2. Effects of supernatants of MNC stimulated with PPD (○) ($n = 4$) or PHA (●) ($n = 3$) on accumulated glucose-stimulated insulin release to the culture medium after 7 days of culture. The results are expressed as percent of the effect of medium.

cillin, 100 mg/l streptomycin and 11.0 mmol/l glucose. For some experiments the lyophilised material was dissolved in isotonic NaCl at a concentration 30 times the original supernatant

Table 2

Effects of cytokines on rat islet function: variation in the reduction in insulin release, insulin content and glucagon content in six different rat islet preparations

Per cent reduction in	1	2	3	4	5	6	Combined results (n = 6) Mean + SEM
Insulin release (ng/10 islets/week)	64 (384/1080)	73 (407/1508)	76 (334/1377)	86 (309/2151)	86 (181/1292)	89 (214/1962)	79 + 4 (305 + 39/1561±178) ^a
Insulin content (ng/islet)	4 (5.0/5.2)	55 (3.8/8.5)	48 (5.0/9.7)	74 (3.2/12.2)	64 (3.2/8.8)	65 (2.8/7.9)	52±10 (3.8±0.5/8.7±1.0) ^a
Glucagon content (ng/islet)	85 (0.05/0.33)	94 (<0.05/0.16)	93 (<0.05/0.46)	95 (<0.05/0.43)	100 (<0.05/0.27)	87 (<0.05/0.23)	92±2 (<0.05/0.32±0.05) ^a

In parentheses are first given the absolute values for islets incubated with cytokines followed by the absolute values for islets incubated without cytokines.

^a Values in islets incubated with cytokines versus values in islets incubated without cytokines: $P < 0.05$.

concentration. These preparations were then stored at -70°C and thawed only once before use.

Culture medium TC 199 with hepes, penicillin, streptomycin and 1 % human serum was dialysed and lyophilized as MNC supernatants and tested with or without PPD or PHA (final concentrations 25 mg/l and 0.25 mg/l, respectively). Only media with a low endotoxin content were used for the production of the MNC supernatants (22).

Assay for activity of leucocyte migration inhibitory factor (LIF)

LIF is elaborated in the G_1 -phase of the cell cycle of activated lymphocytes (23), and the presence of LIF in the supernatants was taken as an indication of successful cytokine produc-

tion. LIF activity was determined by an indirect, two-step, anti-LIF-antibody-controlled migration inhibition assay (2). All active, but no control supernatants had significant LIF activity (2). All supernatants were selected randomly from a panel of LIF-tested materials.

Islet isolation and culture

Islets from collagenase-treated pancreata from male Wistar rats weighing 90–120 g (Møllegaard, Lille Skensved, Denmark), or human cadaveric kidney donors, were isolated and precultured as described in detail elsewhere (4, 16, 20).

After 7 days of preculture the islets were washed once in medium RPMI 1640 with penicillin, streptomycin, hepes, 11 mmol/l glucose, and 0.5 % human serum. Islets from

Table 3

Variation in the effects of cytokines on the function of islets from five human pancreata

		Pancreatic specimen					Combined results ($n = 8$) Mean $\pm$ SEM
		1	2	3	4	5	
Donor age and sex		13 ♂	39 ♀	44 ♀	16 ♀	11 ♀	
Donor HLA-type		A2,24;B5,38; C2;DR6,7	A1,24;B7,15; Cw3,2;DR2,6	A2,24;B39,44; C2;DRw8,-	A1,2;B17,40; C2;DR6,7	A1,29;B8,44; C2;DR3,7	
Warm and cold ischaemia time (min)		23,60	22,80	19,161	unknown	35,80	
Percent reduction in	Cyto- kine batch						
Insulin release	1	52 (289/604)	56 (104/234)	63 (173/465)	NT	NT	48 $\pm$ 8
(ng/10 islets/week)	2	58 (233/554)	24 (249/326)	49 (138/270)	76 (26/103)	4 (137/143)	(169 $\pm$ 30/337 $\pm$ 66) ^a
Insulin content	1	-5 (6.2/5.9)	0 (3.7/3.7)	18 (5.4/6.6)	NT	NT	-7 $\pm$ 17
(ng/islet)	2	-53 (8.4/5.5)	28 (4.1/5.7)	30 (5.2/7.4)	27 ($< 0.8/1.1$)	-100 (1.8/0.9)	(4.5 $\pm$ 0.9/4.6 $\pm$ 0.9) ^b
Glucagon content	1	24 (1.6/2.1)	22 (4.9/6.3)	-23 (5.4/4.4)	NT	NT	7 $\pm$ 15
(ng/islet)	2	0 (2.3/2.3)	15 (5.8/6.8)	39 (4.3/7.0)	53 (0.7/1.5)	-78 (1.6/0.9)	(3.3 $\pm$ 0.7/3.9 $\pm$ 0.9) ^c

Results are expressed as in Table 2.

^a $P < 0.01$; ^b NS; ^c NS.

individual isolates were pooled, mixed, and distributed randomly with 10 islets/ml culture medium RPMI 1640 in plastic Petri dishes (Falcon, Oxnard, USA). The islets were incubated free-floating in 90 % humid atmosphere at 37°C for 7 days in the absence or presence of MNC supernatants, produced either with or without stimulation with PPD or PHA. The final concentration of human serum in all dishes was 0.5 %.

Samples of the medium were taken after 1, 2, 5, 6, and 7 days of culture and analysed for insulin by RIA (8) as standards using rat insulin in experiments with rat islets (NOVO Research Laboratory, Bagsvaerd, Denmark), or porcine insulin in experiments with human islets (Nordisk Gentofte, Denmark). After 7 days of culture the islets were collected, washed once in distilled water, and sonicated for determination of insulin (8) and glucagon (NOVO RIA kit). Some islets were fixed in glutaraldehyde for electron microscopy (14). Cytokine-rich supernatants did not degrade insulin (16).

Statistical analysis

All experiments were set up in duplicate and the average was included in the calculation as one observation. Results are expressed as means ± SEM. A two-tailed paired Wilcoxon's test was used for statistical analysis, except for data shown in Fig. 1. 5 % was chosen as the level of significance.

RESULTS

Effects of cytokines on rat and human islet function

Cytokine-rich MNC supernatants markedly reduced the glucose-induced insulin release from human and rat islets of Langerhans and decreased the contents of insulin and glucagon in rat islets (Tables 2 and 3).

Human islets isolated from different pancreatic specimens varied considerably in their sensitivity to the effects of cytokines compared with different rat islet preparations (Tables 2 and 3).

Table 4
Effects of medium dialysed, lyophilised and reconstituted with PHA or PPD

Medium	Insulin release (ng/10 islets/week)	Insulin content (ng/islet)	Glucagon content (ng/islet)
Untreated (n = 5)	1296 ± 140	12.7 ± 2.0	0.66 ± 0.17
Dialysed and lyophilised (n = 5)	1547 ± 77	13.7 ± 1.3	0.96 ± 0.20
Dialysed and lyophilised + 25 mg/l PPD (n = 5)	1434 ± 94	16.1 ± 2.6	0.76 ± 0.15
Dialysed and lyophilised + 0.25 mg/l PHA (n = 5)	1607 ± 146	11.5 ± 1.6	0.71 ± 0.20

Table 5
Effects of freshly prepared blood mononuclear cell supernatants on islet function

	Medium	Concentration of supernatants of unstimulated MNC			Concentration of supernatants of MNC stimulated with PHA		
		10 %	1 %	0.1 %	10 %	1 %	0.1 %
Insulin release (ng/10 islets/6 days)	2247	1906	2128	1992	193	659	2242
	2385	1703	1893	2000	287	345	1444
Insulin content (ng/islet)	13.3	20.7	15.5	12.7	4.6	14.3	14.7
	14.8	13.4	10.5	10.4	2.8	7.9	9.1
Glucagon content (ng/islet)	0.88	1.45	1.05	0.97	0.25	0.46	0.84
	0.44	0.42	0.36	0.29	0.05	0.05	0.22

Results are shown as means of duplicate set-up in two separate experiments with supernatants from different donors.

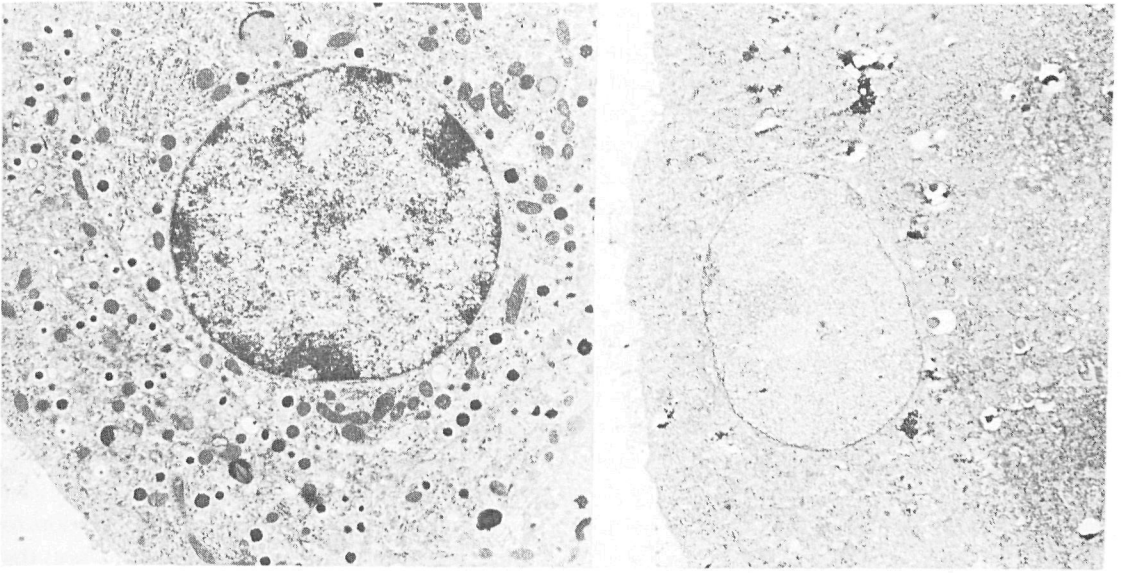


Fig. 3. Representative electron-micrograph of rat islets cultured for 7 days in culture medium (left panel) or in cytokine-rich supernatants at a concentration of 50 % (right panel). Magnification $\times 16,200$.

Dose-dependency of cytokine effects on islet function

The insulin release from rat islets and rat islet content of insulin were not affected by incubation with control supernatants at any concentration (Fig. 1). In contrast, the active supernatants markedly reduced the insulin release and the islet insulin content in a dose-dependent way. At supernatant concentrations above 5 % of the original supernatant concentration, the insulin release was approximately 20 % and the insulin content approximately 40 % of the values found when islets were incubated with control supernatants.

Low concentrations of cytokines, i.e. in high dilutions of active supernatants (0.5 %) or in low dilutions of control supernatants (5 %), increased the islet content of glucagon ($2\alpha =$

0.10 and 0.05, respectively, Wilcoxon's unpaired test, Fig. 1), whereas concentrations of active supernatants above 5 % decreased the glucagon content to about 20 % of the glucagon content found in islets incubated with culture medium.

The dose response curves of the effects of cytokines on insulin release and islet contents of insulin and glucagon closely paralleled each other, i.e. the steepest slope of all curves being between the 1 and 5 % supernatant concentrations.

Supernatants of PPD-stimulated MNC were 10 times more potent than supernatants of PHA-stimulated MNC from the same donor (Fig. 2). From the dose-response curves it can be estimated that the insulin release from rat islets was reduced to 50 % of normal when incubated with supernatants of PPD-stimulated MNC at a final concentration of 4 %, whereas a concentration of 40 % of supernatants of PHA-stimulated MNC was necessary to produce an equal effect. Similar differences in the effects of supernatants of PPD- and PHA-stimulated supernatants were demonstrated on islet contents of insulin and glucagon (data not shown).

Table 6

Effects of endotoxin on rat islet insulin release
(ng/10 islets/6 days) $n = 3$

Medium	2064 $\pm$ 399
Endotoxin 10 mg/l	2199 $\pm$ 160
Endotoxin 1 mg/l	2068 $\pm$ 549
Endotoxin 0.1 mg/l	1979 $\pm$ 187

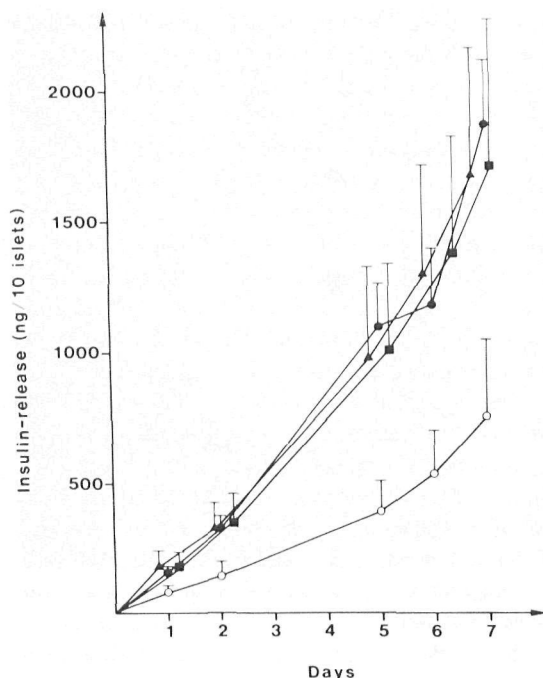


Fig. 4. Glucose-induced insulin release from rat islets incubated for 7 days with either medium RPMI 1640 (●), 50 % supernatant of PHA stimulated MNC: not heated (○), or heated to 56°C for 60 min (■) or to 80°C for 30 min (▲) ($n = 4$).

Control experiments

Incubation of rat islets with culture medium TC 199, which was either dialysed and lyophilised as the MNC supernatants or dialysed, lyophilised and reconstituted with either PPD or PHA, did not decrease the insulin release, the islet content of insulin and glucagon (Table 4), or change islet morphology, when compared with the effect of untreated culture medium.

To study the effects of dialysis and lyophilisation, rat islets were incubated with freshly prepared control and active supernatants of two healthy donors at different concentrations (Table 5). Freshly prepared active supernatants, but not control supernatants, inhibited the insulin release in a dose-dependent way as efficiently as lyophilised active supernatants.

Endotoxin (lipopolysaccharide from *E. coli*,

Endotoxin 186, Malinckrodt, St. Louis, Missouri, USA) in concentrations of 0.1 mg/l–10 mg/l did not affect the insulin release from isolated rat islets (Table 6).

Morphological findings

Light microscopic morphology demonstrated that most islets incubated with cytokine-rich MNC supernatants for 7 days were disintegrated or reduced in size. A few islets in most experiments did not show unequivocal signs of degradation in the light microscope. Electron microscopy of such islets revealed that although the contours of the islets were intact (Fig. 3), the ultrastructure of all types of endocrine islet cells were severely changed with dissolution of organelles and nuclear chromatin. Only the cytoplasmic membrane and the nuclear membrane, as well as a few hormone-containing granules, were preserved.

Physical properties of the islet cell cytotoxic cytokine(s)

Heating of an active supernatant to 56°C for 60 min or 80°C for 30 min completely abolished the inhibitory effect of the insulin release by the untreated active supernatant (Fig. 4).

Control and active supernatants, which were dialysed, lyophilised, and stored at -70°C dissolved at 30 times the original supernatant concentration, did not significantly inhibit the insulin release from rat islets. (Medium: 1635 ± 187 ; control supernatants: 1085 ± 372 , and active supernatants of PHA-stimulated MNC: 1219 ± 369 ng insulin/10 islets/week). Data from the individual five experiments showed no activity in three, but pronounced activity in two of the five batches tested. In contrast, six out of six batches stored as lyophilised material for several months were active when tested repeatedly.

DISCUSSION

Activation of monocytes and lymphocytes by stimulation with antigen or lectin results in the secretion of soluble substances with many dif-

ferent biological activities. Most of these cytokines are antigen-non-specific.

Our hypothesis was that cytokines, as important mediators in the cellular immune response, may participate in cell-mediated destruction of beta-cells leading to Type 1 diabetes mellitus. The first aim was therefore to test the effects of cytokines prepared by stimulation of MNC from healthy, non-diabetic donors, using recall antigen or PHA on isolated islets of Langerhans as targets.

Crude supernatants of stimulated MNC inhibited the insulin release from and decreased the insulin content in isolated islets in a dose-dependent way, indicating an inhibition of insulin biosynthesis, or a cytotoxic effect of soluble factor(s) in these supernatants. Light and electron microscopy showed changes in islet morphology and ultrastructure which were strongly suggestive of a direct cytotoxic effect. This effect was not specific for beta-cells; the glucagon content in the islets was reduced in parallel with the reduction in the insulin release and insulin content, indicating that all islet cells were susceptible to the action of cytokines. However, the tendency to increased islet glucagon content at low cytokine concentrations (Fig. 1) may indicate a difference in the initial action of cytokines on α - and β -cells, as has been demonstrated to be the case with streptozotocin (6).

Cytokine-rich supernatants have been shown to stimulate the activity of various non-lymphoid normal tissues (10, 11, 17, 26). Although a number of cytokines have been shown to arrest the cell cycle of activated lymphocytes, only few have been reported to be cytotoxic. The best characterized cytotoxic cytokines are the lymphotoxins (LTs) and the interferons (IFNs). The former constitute a complex system of toxins comprised of related and unrelated components with molecular weights from 12 KD to more than 200 KD (in humans). They are produced by T- and B-lymphocytes, but their role in lymphocyte-mediated target-cell lysis is not clear. Cells from various tissues and species differ markedly in their sensitivity to LTs, but tumour cells and certain (transformed?) continuous cell lines seem to be particularly sensitive

(7, 9). Thus, it has been suggested that different tissues express different receptors for cytokines as has been shown for LTs (28).

IFNs are glycoproteins with molecular weights between 15 and 25 KD produced by eukaryotic cells in response to viral infections and other stimuli. Thus, sensitized T-lymphocytes elaborate IFN-gamma if challenged with specific antigen, and T-lymphocytes produce the same lymphokine if activated non-specifically, for example by PHA. IFNs inhibit growth of neoplastic and normal cells (12, 27). In this regard, INF-gamma seems to be the most potent species, and in a few cases IFN-gamma has been reported to exhibit direct cytolytic potency (29). This potential, however, may be ascribed to a synergistic effect between IFN-gamma and contaminating LTs (25).

In the present study, supernatants of unstimulated MNC did not impair islet function. In a previous study we found considerable inter-individual and -batch variability in the activity of "unstimulated" MNC supernatants (16). In the previous as well as in the present study, the supernatants were chosen at random from a panel of supernatants. We therefore believe that the batch variation between control supernatants probably reflects inter-individual variation in the constitutive cytokine production by MNC activated *in vivo*, for example as a result of a recent virus infection, or *in vitro* due to variable endotoxin contamination of the culture media, or differences in the mechanical handling of the cells. The dose-response experiments (Fig. 1) support this concept, since very low cytokine concentrations were sufficient to inhibit islet function. Lectin stimulation seems to result in the secretion of cytokines in equal amounts to and with a similar spectrum of biological activities as cytokines produced by antigen stimulation (23). Stimulation of MNC with PPD produced supernatants with a higher activity than those produced by MNC stimulated with PHA. This does not necessarily imply that antigen stimulation leads to a higher cytokine production than lectin stimulation. Thus, PPD, especially in higher concentrations, possesses non-specific mitogenic properties, and PPD also contains small amounts of endotoxin,

which may have potentiated the elaboration of the toxic cytokine(s) (Bendtzen & Baek, unpublished). In any case, the experiments show that the technique used is a highly sensitive *in vitro* method in the detection of quantitative and/or qualitative differences in cytokine activities in MNC supernatants. It will be necessary to evaluate the reproducibility of the *in vitro* islet culture as a semi-quantitative bio-assay by further studies of the variation in the sensitivity of different islet preparations to cytokine effects, as well as studies of the intra- and inter-individual variation in MNC basal and stimulated cytokine-production in healthy donors.

In our opinion, the possibility that the observed effects were the result of a culture artifact can be excluded on the basis of the different control experiments in which dialysed, lyophilised and PPD/PHA reconstituted media were used, as well as freshly isolated supernatants.

Addition of endotoxin to the culture medium did not affect islet function, and the heat sensitivity of the islet-cell cytotoxic factor(s) in the supernatants also excludes the possibility that endotoxin was responsible for the observed effects.

The active component(s) in the supernatants seem(s) to be a heat-sensitive, labile molecule(s) and of a molecular weight above 10 KD, since the supernatant activity on islet function was not lost after dialysis. Further identification of the active substance(s) is the subject of ongoing studies.

There is recent evidence to support the role of MNC secretory products in lymphocyte-mediated target cell lysis (21). On the basis of our findings one could envisage that the selectivity of the beta-cell destruction in Type 1 diabetic patients is most likely due to the homing of specifically sensitised T-effector cells to beta-cell surface antigens; the resulting activation of the T-effector cells leads to the secretion of lymphokines, which recruit and activate antigen-non-specific MNC to build up the insulitis infiltrate. The lymphokines themselves, or the cytokines elaborated by the recruited inflammatory cells, may then damage the adjacent islet cells.

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