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Corynebacterium parvum (Propionibacterium acnes): an inducer of tumor necrosis factor-α in human peripheral blood mononuclear cells and monocytes *in vitro*

The present study investigates the potential capacity of the immunostimulant *Corynebacterium parvum* (C.p.) to induce tumor necrosis factor-α (TNF-α) in human peripheral blood mononuclear cells (PBMC) and blood monocytes (BMo) *in vitro*. Both at the mRNA and protein level, stimulation of PBMC and BMo upon C.p. induces TNF-α. Compared to the hitherto used TNF-α inducers *in vitro* such as Sendai virus, phytohemagglutinin or lipopolysaccharide the C.p. stimulus displayed a threefold stronger induction of TNF-α production ($p < 0.001$). Using C.p. as an inducer it was possible to demonstrate that TNF-α production is regulated by prostaglandin E_2; preincubation of the cells with prostaglandin E_2 resulted in a reduced C.p.-mediated TNF-α production ($p < 0.001$). Co-incubation of interferon-γ (IFN-γ) together with C.p. led to an enhanced release of TNF-α, supporting the assumption that C.p. is a potent TNF-α inducer. The additive effect of IFN-γ and TNF-α on the receptor level was demonstrated by addition of IFN-γ antibodies to the PBMC cultures. Under these conditions TNF-α production, stimulated by C.p. and IFN-γ, was decreased by 30%, compared to the production in assays supplemented with C.p. alone. From these data we conclude that C.p. is a new inducer of TNF-α *in vitro* and a useful tool to study TNF-α production of PBMC and BMo from either healthy donors or from patients.

1 Introduction

TNF was first described by Carswell et al. as a protein found in the serum of primed, LPS-treated animals [1] and is able to produce hemorrhagic necrosis in some murine sarcomas [2]. Further experiments showed that the effects of TNF-α are not limited to inhibition of tumor cell growth, but include a variety of pleiotropic effects, such as immunomodulatory capacities [3–5]. Monocytes (Mo) have been identified as the major producer cells of TNF-α and secrete it upon stimulation with LPS or Sendai virus [6–8]. Despite a large number of studies dealing with the diverse effects of TNF-α, little is known about the role of TNF-α in the regulation of the immune system. Therefore, it is a need to search for those agents that induce TNF-α in human peripheral blood cells *in vitro*. *Propionibacterium acnes* (*Corynebacterium parvum;* C.p.) and several related species of bacteria display strong immunomodulatory effects in mice [9], such as activation of NK cells and MØ [10, 11]. We have recently been able to show that TNF-α activates NK cells in the peritoneal cavity of mice and in human PBMC [12]. In the present study we demonstrate for the first time that C.p. induces TNF-α production in cultures of unprimed human PBMC and blood monocyte (BMo). With this tool in hand it was possible to define the mechanisms by which TNF-α production can be regulated.

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Abbreviations: **(B)Mo:** (Blood) monocytes **C.p.:** *Corynebacterium parvum*

2 Materials and methods

2.1 Cell preparation and culture

PBMC were separated and isolated from heparinized blood by using the standard Ficoll Hypaque sedimentation technique [13]. Isolated cells were washed three times in sterile RPMI 1640 medium (Gibco, Karlsruhe, FRG). Finally, the cells were adjusted to a concentration of 2×10^6/ml culture medium (culture medium: RPMI 1640 supplemented with 10% FCS, 1% amino acids and 1% penicillin/streptomycin, 10 mM Hepes buffer and 1% L-glutamine) and incubated in a humidified incubator at 37 °C and 5% CO_2. Mo were prepared by letting the mononuclear cells adhere to plastic flasks (Greiner, Nürtingen, FRG) for 30–60 min at 37 °C in RPMI 1640 medium with 10% FCS and were recovered by scraping with a rubber policeman after carefully removing nonadherent lymphocytes by several gentle washes with PBS. The procedure was repeated twice to enrich the monocytic cells. The resulting cell population was composed of >95% α-naphthyl-acetate esterase-positive Mo [14, 15]. After the incubation time, SN were spun at 12500 × g for 2 min and were frozen at − 70 °C until the cytokine assay (IFN-α, -γ and TNF-α).

2.2 Reagents

C.p. was purchased from Wellcome, Beckenham, GB. The bacteria suspension was washed three times with RPMI 1640 without antibiotics by centrifugation with 1200 × g for 15 min and was adjusted to 1.4 mg/ml. LPS from *S. typhimurium* was purchased from Sigma (München, FRG). LPS preparations were used at a final concentration of either 1.5 or 10 μg/ml. Antibodies to IFN-α or IFN-γ and to TNF-α as well as preparations of IFN-α, -γ or TNF-α were

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kindly provided by Hoffmann-La Roche, Basel, Switzerland. PGE_2 (applied at a final concentration of 5×10^{-7} M) was obtained from Sigma. Sendai virus was kindly provided by Prof. Rott, Giessen, FRG. The virus was used at a final concentration of 5×2^9 HAU/ml. Vesicular stomatitis virus (VSV) was donated by Prof. Kirchner, (Lübeck, FRG) and was used at 5×10^5 PFU/ml. Con A (6,25 μg/ml) and PHA (6,25 μg/ml) were obtained from Wellcome and from Pharmacia (Uppsala, Sweden), respectively.

2.3 Immunoassays for IFN and TNF-α

IFN-α, -γ and TNF-α levels were determined by ELISA as previously described [14, 16, 17]. Briefly, for the determination of TNF-α levels, microtiterplates (96 wells) were coated with 5 μg/ml of murine anti-TNF-α mAb for 24 h, washed twice with distilled water blocked with buffer solution containing 10 g/l BSA in 0.2 M Tris-HCl, pH 7.5, and kept at 2–8 °C before use. Diluted serum samples or cell SN were added to the wells and compared with serial dilutions of rTNF-α (sp. act. 2×10^7 U/mg) after adding 100 ng/ml peroxidase-labeled rabbit anti-TNF-α antibodies and incubating for 24 h at 2–8 °C. Absorbance readings at 450 nm were made within 10 min of addition of the peroxidase substrate. The ELISA was sensitive to TNF-α concentrations > 45 pg/ml and showed no cross-reaction with LPS, IFN-α or -γ, TNF-β, IL 1α or -β and IL 2.

2.4 Bioassay for TNF-α

Biological activity of TNF-α in SN from cell cultures was quantitated in 96-well plates by a cytotoxicity assay using L929 cells [14, 18]. Briefly, 5×10^4 cells were seeded in each well and incubated for 24 h at 37 °C in RPMI 1640 medium containing 10% FCS. Then culture SN were decanted and 100 μl of actinomycin D (Sigma; 15 μg/ml) and 100 μl of RPMI medium with 10% FCS were added to each well. After adding 100 μl of medium (= control), TNF-α standard or samples, the assays were reincubated for one day. Then the cells were stained with cristal violet and washed. The absorbance was measured in an ELISA reader at 595 nm and the percentage of cytotoxicity was determined according to the formula:

$$\% \text{ Cytotoxicity} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

One unit of cytotoxicity was defined as the concentration at which 50% of the L-929 cells showed cytopathic effects after 24 h. Human rTNF-α (sp. act.: 2×10^7 U/mg) was used as a standard.

2.5 DNA probes

Human TNF-α cDNA, a 620-bp fragment (Xho I-Hind III fragment) was cloned into pUC8 [19]; the human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was cloned into pUC 19 [20]. The cDNA probes were nick translated with [α-$_{32}$P]dTTP to a specific radioactivity of 5×10^7–7×10^7 cpm/μg DNA [21].

2.6 RNA preparations and Northern blot hybridization

Total RNA of the cells was isolated according to the thiocyanate extraction procedure [22]. Subsequently, poly(A)$^+$-rich mRNA was isolated from total RNA by oligo(dT)-cellulose chromatography [23]. The concentration of the extracted RNA was calculated on based on 1 unit of absorbance at 260 nm = 37.2 μg RNA/ml.

For Northern blot hybridization, poly(A)$^+$-rich mRNA was denatured at 56 °C for 30 min in electrophoresis buffer (40 nM Mops, 10 mM sodium acetate, 1 mM EDTA, pH 7.2) containing 50% DMSO and 6% formaldehyde and subjected to electrophoresis on 1.1% agarose gels containing 6% formaldehyde [24]. The separated RNA were blotted on nitrocellulose and hybridized with the ^{32}P-labeled probes [24]. The dry nitrocellulose filters were exposed to Kodak XAR-5 X-ray film (Eastman Kodak, Rochester, NY) with one intensifying screen for 3 days at −70 °C.

2.7 Statistical evaluation

Results were expressed as mean and SD (x ± SD) and statistical significance (n.s. = not significant, $p < 0.01$ and $p < 0.001$) was analyzed by Student's t-test [25]. The statistical analysis was performed on a Macintosh SE/30 computer (Apple Computer Inc., Cupertino, CA) using StatView software (Abacus Concepts Inc., Berkeley, CA).

3 Results

3.1 *In vitro* production of TNF-α by PBMC in response to C.p. stimulation

C.p. was added at various doses to PBMC (2×10^6 cells/ml). After overnight incubation, SN were collected and tested for TNF-α content by ELISA. As can be seen in Fig. 1, maximum TNF-α production was obtained after stimula-

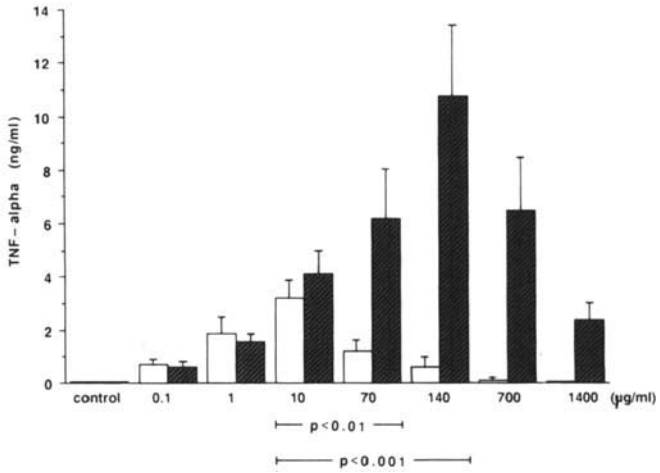

Figure 1. Production of TNF-α upon stimulation with C.p. (■) and LPS (□) in PBMC (2×10^6 cells/ml) of healthy donors ($n = 26$). SN were collected after 24 h of incubation and TNF-α was determined by an ELISA with a sensitivity of 45 pg/ml. The levels are given in ng/ml. Data are shown as mean ± SD and significance is indicated.

tion with 140 μg/ml of C.p. Since concentrations up to 1400 μg/ml did not produce significantly higher titers of TNF-α, we routinely used 140 μg/ml. Compared to LPS-mediated TNF-α production, significant differences could be obtained at 10 to 70 μg/ml. In response to C.p. stimulation, a more than threefold elevated synthesis of TNF-α could be observed.

3.2 Kinetics of TNF-α production upon C.p. stimulation

As can be seen in Fig. 2, TNF-α protein production was already detectable after 2–5 h of incubation of human PBMC (2×10^6 cells/ml) *in vitro*. Maximal titers were obtained after 20–24 h of incubation of the cells in the presence of 140 μg/ml C.p. Incubation time up to 80 h showed no further enhancement of TNF-α production. Kinetics of TNF-α synthesis in response to LPS (10 μg/ml) revealed similar results with significant differences for the absolute amount of the cytokines.

3.3 Comparison of C.p. with other TNF-α inducers

In order to determine whether C.p. is more active in inducing TNF-α than other known powerful TNF-α-inducing agents, C.p. was compared to Sendai virus

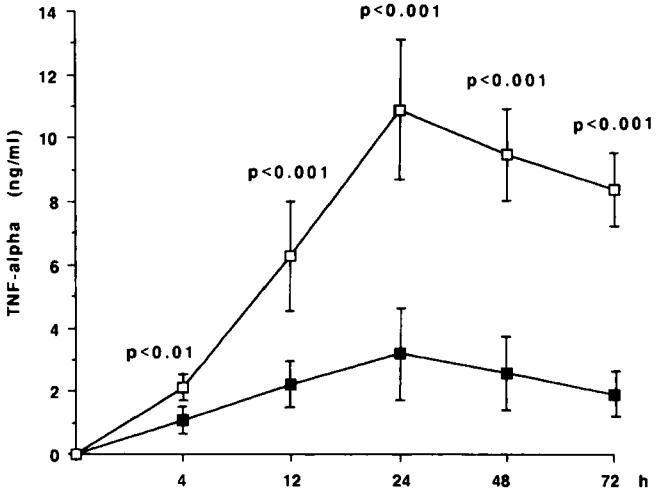

Figure 2. Kinetics of TNF-α production in PBMC (2×10^6 cells/ml) of healthy donors ($n = 34$) in response to stimulation with C.p. (140 μg/ml) and LPS (10 μg/ml), respectively. The data represent mean values ± SD of TNF-α and significance is given.

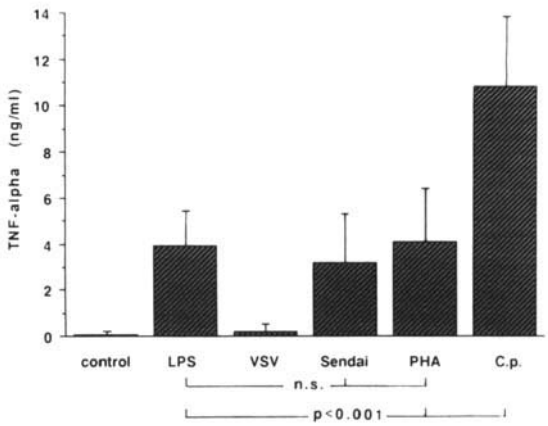

Figure 3. Comparison of different inducers of TNF-α in human PBMC (2×10^6/ml) after 24 h of incubation (healthy donors, $n = 19$). Measurement of TNF-α levels (ng/ml) was done by ELISA. TNF-α-inducing agents were used as follows: LPS (10 μg/ml), VSV (5×10^5 PFU/ml), Sendai virus (2^9 HAU/ml), PHA (6.25 μg/ml) and C.p. (140 μg/ml). The data are shown as mean ± SD and the significance is given (n.s. = not significant, $p < 0.001$).

(2^9 HAU/ml), three different preparations of LPS (10 μg/ml) and PHA (6.25 μg/ml) in human PBMC *in vitro*. As indicated in Fig. 3, C.p. induces significant higher titers of TNF-α than LPS or Sendai virus. No IFN-α and only little amounts of IFN-γ (< 0.5 U/ml) could be detected after incubation of PBMC with C.p. as measured by ELISA [14, 15], (data not shown).

3.4 Characterization of TNF-α in peripheral blood cells *in vitro*

Different cell types respond to adequate stimulus by secreting TNF-α, but Mo/MΦ are especially known to synthesize large amounts of the protein in cell culture SN. We used purified BMo to evaluate their capacity of TNF-α production upon C.p. stimulation after 24 h of incubation. As shown in Table 1, BMo induction yielded a 30%–40% enhanced TNF-α synthesis compared to PBMC, as measured by ELISA. Preincubation with a polyclonal rabbit anti-TNF-α antiserum in excess showed blocking of the assay procedure. In contrast to the measurement of antibody-antigen complex formation in the immunoassay, the TNF-α bioassay is able to determine the functional properties of the cytokine. Results of TNF-α measurement showed only slight differences compared to those obtained

Table 1. Production of TNF-α by PBMC and BMo[a)]

	PBMC		BMo	
	ELISA	Bioassay	ELISA	Bioassay
	TNF-α (ng/ml)			
Control, $n = 21$	< 0.1	< 0.6	< 0.2	< 0.8
C.p.[b)], $n = 19$	10.8 ± 2.6	9.4 ± 5.5	14.9 ± 3.8	12.6 ± 7.6
C.p.[b)] + anti-TNF-α, $n = 14$	< 0.5	< 1.0	< 0.6	< 1.0

a) PBMC (2×10^6/ml) and BMo (2×10^6/ml) upon stimulation with C.p. for 24 h of incubation. Blocking experiments with polyclonal anti-human-TNF-α antibodies were performed after preincubation with the antibody in excess for 1–2 h at room temperature. SN were collected and tested for TNF-α content both by ELISA and bioassay. Data are given as mean values ± SD. n = number of healthy donors tested.

b) 140 μg/ml.

Table 2. PGE_2-mediated regulation of C.p.-induced TNF-α production[a)]

Exp. no	C.p.[b)]	C.p.[b)] + PGE_2[c)] TNF-α (ng/ml)	C.p.[b)] + PGE_2[d)]	C.p.[b)] + PGE_2[e)]
1	14.3	0.4	2.8	7.8
2	11.8	0.7	2.4	6.2
3	12.4	0.1	2.1	3.0
4	10.8	0.1	1.2	5.4
5	10.5	1.4	2.1	5.7
6	13.3	3.1	4.7	8.1
7	11.0	0.8	2.7	7.3
8	7.8	0.4	1.8	2.1
9	12.8	2.4	3.2	2.9
10	10.2	0.2	0.8	6.1
X ± SD:	11.5 ± 1.8	1.0 ± 1.1 $p < 0.001$	2.4 ± 1.0 $p < 0.001$	5.5 ± 2.1 $p < 0.01$

a) PBMC (2×10^6/ml) from 10 healthy donors were stimulated for 24 h with C.p. (140 μg/ml) alone in the control group, whereas the cells of the experimental group were preincubated with various doses of PGE_2 for 1 h. Experiments were done in triplicates. Mean values ± SD and significance are shown.
b) 140 μg/ml.
c) 5×10^{-5} M.
d) 5×10^{-6} M.
e) 5×10^{-7} M.

by ELISA (not significant). Furthermore, the blocking capacity of the polyclonal TNF-α antibodies was confirmed in the bioassay.

3.5 TNF-α gene expression in PBMC and BMo

In order to elucidate whether the TNFα synthesis at the protein level is paralleled by mRNA production, transcription of the TNF-α gene and its inducibility by C.p. and LPS were analyzed by Northern blot analysis both in PBMC (2×10^6 cells/ml) and BMo (2×10^6 cells/ml) after 24 h of incubation. Fig. 4 shows the absence of TNF-α transcripts in untreated cells (lane e), only the GAPDH mRNA could be detected as a reference transcript. BMo respond to stimulation with either LPS (lane a) or C.p. (lane c) at optimal concentration with clearly detectable TNF-α mRNA production. Slightly reduced signals could be observed in PBMC, treated under the same conditions.

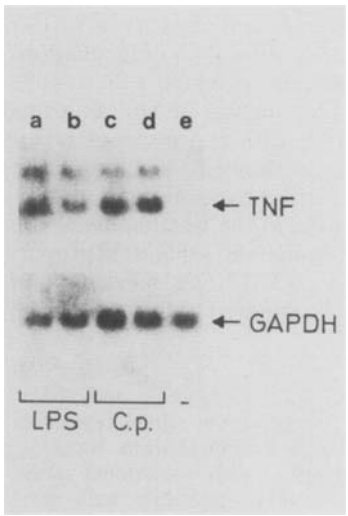

Figure 4. Northern blot analysis of TNF-α transcripts after stimulation of BMo (a and c) and PBMC (b and d) with C.p. (140 μg/ml) and LPS (10 μg/ml) in comparison to unstimulated cells. Cells (2×10^6/ml) were incubated for 24 h and RNA was isolated as described in Sect. 2.6. Aliquots of 2 μg of RNA were loaded in each lane; the Northern blot was hybridized with the ^{32}P-labeled TNF-α and GAPDH probes.

3.6 Regulation of TNF-α production by PGE_2

To examine whether PGE_2, which is involved in inflammation, takes part in the regulation of TNF-α production, the cells were incubated with different doses of PGE_2 and C.p. Table 2 shows that in this experimental situation TNF-α production is significantly reduced in a dose-dependent manner. Thus, it appears that PGE_2 plays a major role in the regulation of TNF-α induction.

3.7 Synergistic effects between TNF-α and IFN-γ

It has been described that TNF-α and IFN-γ cause synergistic cytotoxic effects [26, 27]. Therefore, we examined whether TNF-α production is enhanced after stimulation of cells with C.p. and/or IFN-γ. As summarized in Table 3, TNF-α production was higher in experiments after co-incubation with C.p. and IFN-γ, compared to stimulation

Table 3. Induction of TNF-α in PBMC of healthy donors ($n = 23$) upon stimulation with C.p. (140 μg/ml) and IFN-γ (100 U/ml), respectively[a)]

Inducer	TNF-α (ng/ml)
C.p.	10.8 ± 2.6
C.p. + anti-IFN-γ	7.9 ± 2.4
IFN-γ	2.8 ± 1.2
IFN-γ + anti-IFN-γ	0.3 ± 0.1
C.p. + IFN-γ	13.6 ± 4.2
C.p. + IFN-γ + anti-IFN-γ	9.6 ± 3.4

a) Data are presented as mean values ± SD. Experiments with polyclonal rabbit anti-IFN-γ antibodies were performed after preincubation with the antibodies in excess for 1–2 h at room temperature.

with either C.p. or IFN-γ alone. Receptor cross-reactivity is a common hypothesis for synergistic reactivity between IFN-γ and TNF-α [28]. Therefore, PBMC were incubated with antibodies to IFN-γ. The results revealed that TNF-α production was reduced up to 30% compared to samples that were incubated with C.p. alone (Table 3).

4 Discussion

C.p. is a strong stimulant of the lymphoreticular system and has been successfully used for immunotherapy of experimental tumors in animals and humans [1–5]. C.p. has also been reported to enhance TNF-α production *in vivo* in mice when injected together with LPS [2, 28]. However, no data were available indicating that C.p. can directly activate TNF-α production in isolated human PBMC or BMo in the absence of any prestimulation by other immunomodulators. Our data show that C.p. is a powerful inducer of TNF-α over a wide range of concentrations both at the transcriptional and translational level. TNF-α production caused by C.p. is even threefold higher than that obtained with the classical TNF-α inducer *LPS*. The following two lines of evidence were presented which indicate that C.p. induced TNF-α production directly and not indirectly via IFN-γ induction: (a) it was demonstrated that IFN-γ, added either exogenously or produced endogenously via mitogen or virus induction, caused only a low TNF-α production. Blocking experiments with IFN-γ antibodies in addition to C.p. stimulation showed only slight reduction of the resulting TNF-α synthesis and (b) addition of C.p. together with IFN-γ yielded only an additive effect on TNF-α production *in vitro* compared to the effect of C.p. and IFN-γ alone. Hence, C.p. is another inducer of TNF-α *in vitro* which is superior to the well known TNF-α inducer IFN-γ [29, 30]. Moreover, the data presented indicate that the C.p.-induced TNF-α production is regulated by PG of the E series. This conclusion was drawn from the studies which revealed that co-incubation of PGE_2 together with C.p. leads to a reduced TNF-α production *in vitro*. Experiments are currently performed to elucidate whether a similar control mechanism regulates TNF-α production *in vivo*. In some previous studies the observed anti-tumor effect of C.p. has been attributed to low IFN induction [10]. However, in view of the present data it appears to be more likely that this *in vivo* effect should mainly be attributed to an enhanced TNF-α production.

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