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Tachykinin activation of human monocytes from patients with rheumatoid arthritis: in vitro and ex-vivo effects of cyclosporin A

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Summary Three types of tachykinin receptors, namely NK_1, NK_2 and NK_3, are known to preferentially interact with substance P (SP), neurokinin A (NKA) and neurokinin B (NKB), respectively. We previously demonstrated that NK_1 and NK_2 receptors are present on human monocytes, SP and NKA inducing superoxide anion production and tumor necrosis factor- alpha (TNF-α) mRNA expression. NK_2 receptor stimulation also triggered an enhanced respiratory burst in monocytes isolated from rheumatoid arthritis (RA) patients. This study was aimed to evaluate the in vitro and ex-vivo effects of cyclosporin A (CsA) on tachykinins-evoked TNF-α release from monocytes of healthy donors and RA patients. CsA (100 ng/ml) potently inhibited phorbol ester- and tachykinin-evoked TNF-α secretion. In RA patients treated with CsA (Sandimmun^R Neoral^R) 2.5 mg/kg/die, a significant time-dependent reduction in TNF-α secretion from monocytes was measured. This may contribute to the CsA therapeutic activity in RA. © 2001 Harcourt Publishers Ltd

INTRODUCTION

Rheumatoid arthritis (RA), a common systemic disease of unknown origin, is characterized by a chronic inflammation of the synovial joints with infiltration and accumulation of activated blood cells, mainly memory T-cells, monocytes/macrophages and plasma cells. This leads to the progressive destruction of cartilage and bone (largely mediated by cytokines, secreted by T-lymphocytes and monocytes) and the development of joint deformities (Feldmann et al., 1996a; Brunelleschi, 1999). A role for tachykinins in the onset and development of RA stems from different experimental and clinical observations (Brunelleschi, 1999). In animals, intraneuronal substance P (SP) contributes to the severity of experimental arthritis, denervation of SP nerve fibers by capsaicin delaying the onset, reducing the intensity of the symptoms and also preventing controlateral arthritic changes (Levine et al., 1984; Felten et al., 1992). Elevated levels of tachykinin-like immunoreactivity in the joint fluid as well as a loss of neuropeptidergic innervation in the synovium have been detected in RA patients (Devillier et al., 1986; Pereira Da Silva and Carmo-Fonseca, 1990). The prominent role played by SP in nociception and the ability of SP and neurokinin A (NKA) to modulate the activity of those cell populations (e.g. T- and B-lymphocytes, monocytes, macrophages, synoviocytes) involved in RA, might also suggest tachykinin receptor antagonists as possible potential therapeutics for RA (Brunelleschi, 1999). Previous observations revealed that human peripheral blood monocytes as well as the murine macrophage line P388D1 possess tachykinin NK_1 receptors and also produce SP (Ho et al., 1997; Pascual and Bost, 1990). By using natural tachykinins and selective receptor agonists and antagonists, we demonstrated that NK_1 and NK_2 receptors are present on human monocytes, their stimulation leading to superoxide anion production and increased tumor necrosis factor-alpha (TNF-α) mRNA expression (Brunelleschi et al., 1998). We also showed that monocytes isolated from RA patients present a greater sensitivity to tachykinin effects, NK_2 receptor stimulation triggering an enhanced respiratory burst (Brunelleschi et al., 1998). Moreover, human monocytes are known to release TNF-α and other inflammatory cytokines, when challenged in vitro with nanomolar concentrations of SP and

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NKA (Lotz et al., 1988). TNF-α is a pleiotropic cytokine that plays a key role in RA, being at the apex of a pro-inflammatory cytokine cascade; it can also represent a good therapeutic target, since blockade of TNF-α reduced the effects of many other pro-inflammatory mediators (Feldmann et al., 1996 a,b). As a matter of fact, TNF-α antagonists (e.g. etanercept) and chimeric anti-TNF-α monoclonal antibodies (e.g. infliximab) are recognized as safe and effective agents for short-term therapeutic use in RA (Jones and Moreland, 1999; Maini et al., 1999). Cyclosporin A (CsA), a cyclic polipeptide produced as a metabolite by the fungus species *Beauveria nivea*, is an immunosuppressant drug used for the treatment of RA (Cush et al., 1999; Altman et al., 1999). It has been reported to delay radiological disease progression and also to inhibit joint damage deterioration in early RA patients (Drosos et al., 2000). CsA binds to a family of basic cytosolic receptor proteins, named cyclophilins. The CsA-cyclophilin complex, on its turn, binds to, and inhibits, the activity of the calcium/calmodulin-dependent serine/threonine protein phosphatase 2B, calcineurin (Liu, 1993; Ho et al., 1996). Inactive calcineurin is unable to activate the nuclear factor of activated T cells (NF-AT), a transcription factor required for the expression of interleukin 2 (IL-2) gene in lymphocytes (Liu, 1993). Moreover, CsA has also been shown to inhibit lipopolysaccharide (LPS)- induced NF-κB (nuclear factor-kappa B) activation and to interfere with the inducible degradation of NF-κB inhibitors (Holschermann et al., 1996; Marienfeld et al., 1997). Although its precise mechanism of action in RA is not fully elucidated (Bentin, 1995), CsA blocks T- lymphocyte activity, type 1 T-helper cells (Th1) being preferentially suppressed (Schreiber and Crabtree, 1992; Schmidt et al., 1994). Furthermore, it inhibits HLA-DR expression (Yonish-Rouach et al., 1991), reduces TNF-α and IL-1β production (Andersson et al., 1992; Schmidt et al., 1994) in human monocytes. CsA has been also demonstrated to induce apoptosis in immature and cultured monocytes, but not in synovial macrophages (Cutolo et al., 1998). Recently, a significant decrease in circulating TNF-α has been documented in RA patients treated with CsA for 16 weeks (Kim et al., 2000).

Therefore, we decided to evaluate the link (if any) and the reciprocal interactions between tachykinins, TNF-α and CsA in RA.

To do so, we checked CsA ability to interfere, in vitro, with TNF-α release from monocytes isolated from healthy donors and stimulated by SP, NKA and [β-Ala^8]-NKA (4–10), a selective NK_2 agonist. Tachykinins effects were also compared with those elicited by the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA), a well known standard stimulus for monocytes. We also evaluated the ex-vivo effects of CsA by testing monocytes obtained from six RA patients treated with this drug.

MATERIALS AND METHODS

Isolation of peripheral blood monocytes

Peripheral blood monocytes were isolated from heparinized venous blood (30–40 ml) by standard techniques of dextran sedimentation, Ficoll-Paque (density = 1.077 g/cm^3) gradient centrifugation (400 × *g*, 30 min, room temperature) and recovered by thin suction at the interface (Brunelleschi et al., 1998). Cells were then washed twice with phosphate- buffered saline (PBS, pH 7.4) and resuspended at $1–2 \times 10^7$/ml in RPMI 1640 medium, supplemented with 5% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, 50 μg/ml streptomicin and 5 U/ml penicillin. Cell viability, as assessed by trypan blue dye exclusion, was > 98%. Purified monocyte populations were obtained by adhesion (see below) and also assessed by flow citometry with monoclonal antibodies anti-CD14 and anti-CD3, monocyte populations routinely consisting of > 80% CD14$^+$ cells and < 3% CD3$^+$ cells. Briefly, 100 μl of cell suspension were plated in six-well tissue culture plates (35 mm diameter, Costar, UK) and allowed to adhere for 90 min at 37°C in a humidified atmosphere containing 5% CO_2. Non-adherent cells (mainly lymphocytes) were removed by three gentle washings with warm PBS. In some experiments, monocytes were obtained from RA patients treated with cyclosporin A (Sandimmun^R Neoral^R, Novartis Farma; see below).

Monocyte culture and measurement of TNF-α release

Adherent monocytes ($0.6–1 \times 10^6$/plate), isolated from both healthy donors and CsA-treated RA patients, were cultured in RPMI medium supplemented as above except for FCS. In experiments aimed to evaluate the in vitro effects of CsA, monocytes from healthy donors were treated with or without cyclosporin A (CsA; 100 ng/ml) for 16 h at 37°C in a 5% CO_2 incubator and then stimulated with optimal concentrations of phorbol 12-myristate 13-acetate (PMA; 10^{-7}M), substance P (SP; 10^{-7}M), neurokinin A (NKA; 10^{-7}M) or the NK_2 selective agonist [β-Ala^8]-NKA(4–10) (10^{-7}M). After a 24 h incubation at 37°C in 5% CO_2, culture supernatants were harvested and stored at −20°C until assay. In experiments aimed to evaluate ex-vivo CsA effects, monocytes were isolated from six RA patients at different times (see below: **Patients**) and stimulated with PMA, SP, NKA or the NK_2 selective agonist for 24 h as above. PMA, NKA and the NK_2 receptor agonist were dissolved in DMSO; SP was dissolved in water. CsA (10 mg) was dissolved in 0.5 ml ethanol/Tween mixture (9:1) and diluted in 0.5 ml water to give a 10 mg/ml concentration.

TNF-α in the samples was measured using enzyme-linked immunoassay kit (Pelikine Compact^{TM} human TNF-α ELISA kit). The measurements were performed

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according to manufacturer's instructions (CLB, Central Laboratory of the Netherlands Red Cross, Netherlands). The minimum detectable concentration of human TNF-α was <1.4 pg/ml. No cross-reactivity was observed with any other known cytokine. Control values (e.g. TNF-α release from untreated, unstimulated monocytes) were subtracted from all determinations. Results are means $\pm$ s.e.m. of duplicate determinations and are expressed in pg/ml. Since unstimulated monocytes from RA patients released *per se* very high amounts of TNF-α, results are also expressed as percent of control value. Statistical analysis was performed by Student's *t* test for paired or unpaired samples.

Patients

Six patients, two males and four females, aged between 38 and 70 years, with a newly diagnosed rheumatoid arthritis were studied. Blood (about 30 ml) was withdrawn at 8.00 on the first day of the study (T_0, before the first administration of CsA), after two (T_1) and four (T_2) weeks of CsA treatment. All patients gave their informed consent and, after the first day of the study (T_0; when blood was withdrawn to measure baseline TNF-α release), received cyclosporin A (Sandimmun^R Neoral^R, Novartis Farma, Italy) per os at a daily dose of 2.5 mg/kg. The study and the research protocol were approved by the local Ethical Committee.

Chemicals

The compounds used and their sources were as follows: dextran T-500 and Ficoll-Paque (Pharmacia, Sweden); PBS, RPMI 1640 medium, FCS, glutamine, streptomicin, penicillin and PMA (Sigma, Italy); fluorochrome-conjugated anti-CD14 and anti-CD3 (Becton Dickinson, UK); SP, NKA and [β-Ala^8]-NKA(4–10) (Peninsula, UK). Cyclosporin A for in vitro study was obtained from Novartis Pharma AG (Switzerland).

RESULTS

Effects of cyclosporin A on TNF-α release by monocytes isolated from healthy donors

Unstimulated monocytes (1×10^6 cells) isolated from healthy donors released 4 ± 1 pg/ml TNF-α ($n = 6$); this value was subtracted from all determinations.

PMA, SP, NKA and the NK_2 selective agonist [β-Ala^8]-NKA (4–10), all evaluated at 10^{-7}M, induced TNF-α release from human monocytes (Fig. 1A). This concentration was selected according to data from the literature (Lotz et al., 1988) and is consistent with our observations on superoxide anion production from monocytes, where tachykinin effects were maximal at 10^{-7}M (Brunelleschi et al., 1998). SP, NKA and the selective NK_2 agonist released similar amounts (41.7 ± 14, 43 ± 11 and 45 ± 11 ng/ml, respectively) of TNF-α ($n = 6$; Fig. 1A) whereas the release of TNF-α induced by PMA was significantly higher (963 ± 124 pg/ml; $n = 6$; $P < 0.001$) (Fig. 1A). Cyclosporine (CsA) inhibited, in a dose-dependent (0.01–500 ng/ml) manner, TNF-α release from human monocytes (data not shown). At 100 ng/ml, a concentration in the pharmacological range employed for RA treatment (Cutolo et al., 1998), CsA inhibited to $45 \pm 2\%$ ($n = 6$) PMA-evoked TNF-α release and almost completely abolished (about 90% inhibition) the tachykinins-evoked ones (Fig. 1B).

Ex-vivo effects of cyclosporin A (Sandimmun^R Neoral^R) on monocytes from rheumatoid arthritis patients

Monocytes were isolated at three different times (T_0: before the first administration of Sandimmun^R Neoral^R; T_1: after two weeks of drug treatment; T_2: after four weeks of drug treatment) from six rheumatoid arthtritis patients, who were given CsA (Sandimmun^R Neoral^R) 2.5 mg/kg/day.

Monocytes obtained from RA patients release higher amounts of TNF-α (Leirisalo-Repo et al., 1995) and RA patients present increased serum levels of different cytokines, TNF-α being among the more elevated ones (Kim et al., 2000); so, it was not surprising that monocytes isolated from RA patients spontaneously secreted relatively high amounts of TNF-α. These measured TNF-α amounts were: 213 ± 76 pg/ml (range, 106–591) at T_0; 312 ± 92 pg/ml (range, 91–610) at T_1 and 119 ± 26 pg/ml (range, 33–199) at T_2. These values were subtracted from those obtained with the stimuli.

As depicted in Figure 2A, PMA-induced TNF-α release from human monocytes dropped from 1264 ± 250 pg/ml to 513 ± 265 pg/ml (about 60% inhibition) after two weeks of CsA treatment, and was further reduced to 312 ± 49 pg/ml (more than 70% inhibition vs baseline values) after one month of therapy (Fig. 2A; $n = 6$). Tachykinins-evoked TNF-α release from RA monocytes was similar reduced by CsA treatment (Fig. 2B). Interestingly, baseline values (T_0, that is, before CsA treatment) of tachykinin-evoked TNF-α release were higher ($P < 0.05$) than those obtained from monocytes isolated from healthy donors. Moreover, CsA treatment significantly reduced, in a time-dependent (about 50–60% inhibition at T_1 and 80–90% inhibition at T_2) manner, tachykinins-evoked TNF-α secretion from RA monocytes, with minimal variations among the three tachykinins used (Fig. 2B). In these experiments, SP, NKA and [β-Ala^8]-NKA(4–10) were evaluated at two concentrations, 10^{-7}M, as for control cells, and 10^{-9}M. This was done because we previously demonstrated that RA monocytes present an enhanced in vitro sensitivity to NK_2 receptor stimulation and the NK_2 selective agonist evoked a near maximal superoxide anion production at 10^{-9}M (Brunelleschi et al., 1998). CsA had no effect on the

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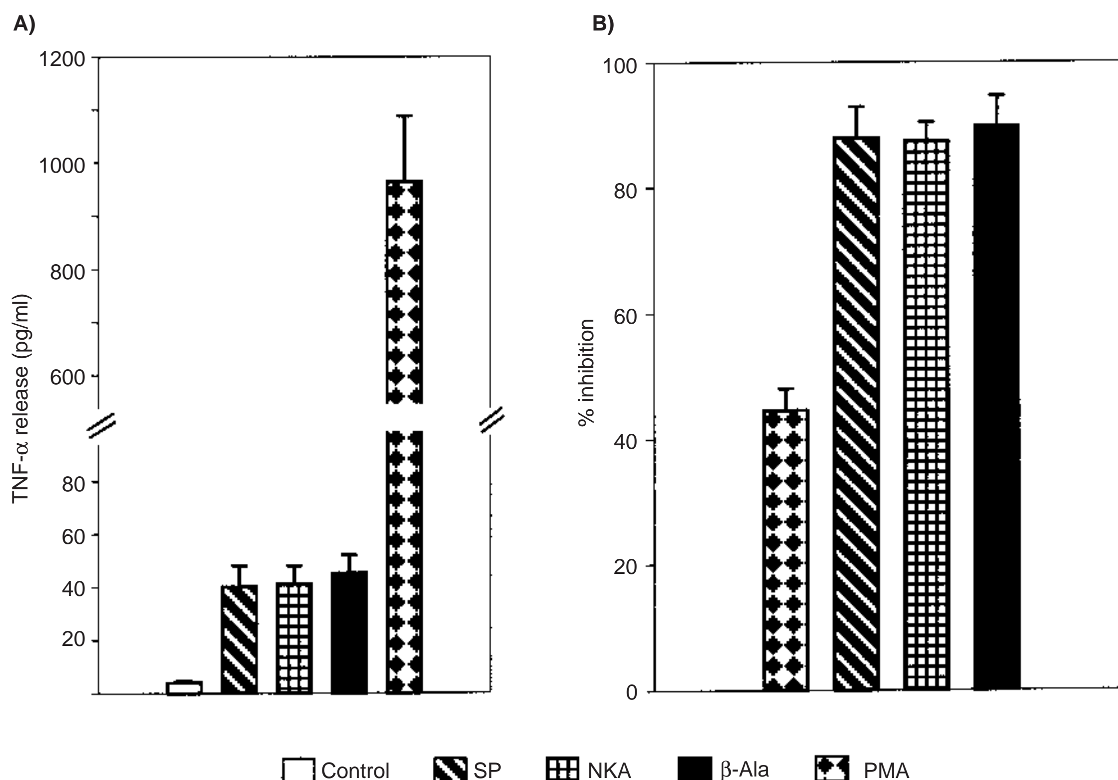

Fig. 1 CsA inhibits TNF-α secretion from PMA- or tachykinins-stimulated monocytes. A) TNF-α secretion from control or stimulated monocytes. Cells (1×10^6 monocytes) were challenged with vehicle or PMA, SP, NKA or the selective NK_2 receptor agonist [β-Ala[8]]-NKA(4–10), all at 10^{-7}M, for 24 h. B) Inhibitory effects of CsA on PMA- or tachykinins-evoked TNF-α secretion. Cells were treated with CsA 100 ng/ml or vehicle for 16 h and then challenged with stimuli for a further 24 h. Means + s.e.m. of 6 experiments.

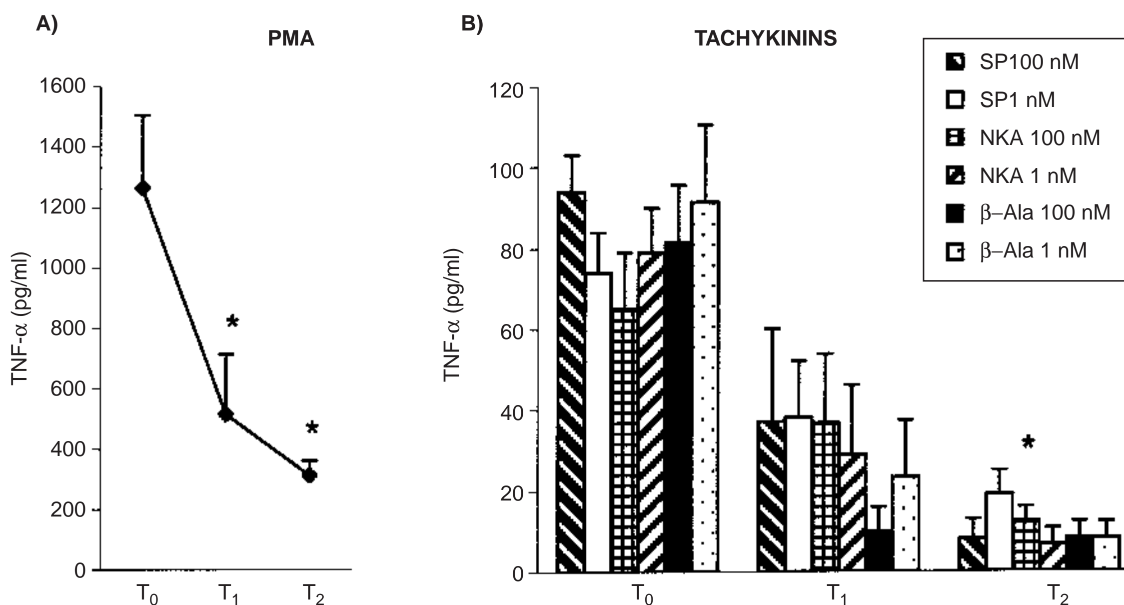

Fig. 2 TNF-α secretion from monocytes isolated from RA patients: effects of CsA (Sandimmun[R] Neoral[R]) treatment. Monocytes were collected at different times: T_0 (before the first CsA administration), T_1 (after two week-treatment) and T_2 (after four-week treatment). A) PMA-evoked TNF-α secretion from monocytes of RA patients treated with CsA. B) Tachykinin- evoked TNF-α secretion from monocytes of RA patients treated with CsA. Cells were challenged with PMA or tachykinins (all at 10^{-7}M) for 24 h. Results are means + s.e.m. of 6 experiments. See Methods for further details. * $P < 0.05$.

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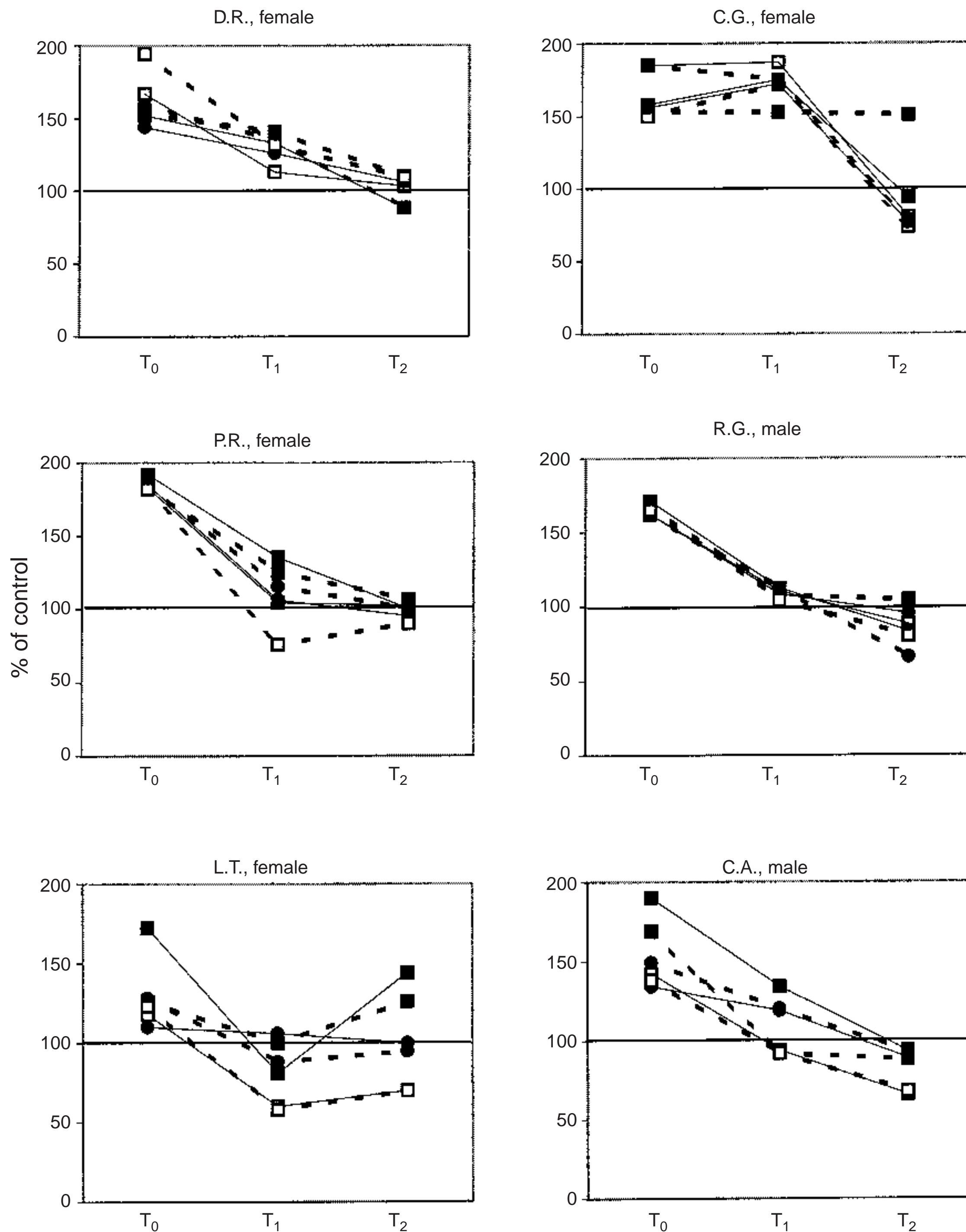

Fig. 3 Tachykinins-evoked TNF-α secretion from monocytes of RA patients treated with CsA (Sandimmun[R] Neoral[R]). The pattern of cytokine secretion decrease is depicted for each one of the six patients evaluated. See Methods for further details. SP 100 nM: ■—■; SP 1 nM: ■- - -■; NKA 100 nM: ●—●; NKA 1 nM: ●- - -●; [β-Ala[8]]-NKA (4–10) 100 nM: □—□; [β-Ala[8]]-NKA (4–10) 1 nM: □- - -□. * $P < 0.05$.

respiratory burst (either in vitro or ex-vivo) from monocytes (data not shown), consistently with previous observations in human neutrophils (Chiara et al., 1991),

As detailed before, baseline TNF-α secretion from RA monocytes differed significantly among patients. Figure 3 illustrates the individual TNF-α release along with time for each patient, a significant reduction from values measured at T_0 being observed at the end of the study in all cases. In this figure, tachykinins-evoked TNF-α release (similar data were also obtained with PMA; not shown) is

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expressed as percent of control value, the value obtained from unstimulated monocytes being 100% for each one of the six RA patients who entered this study.

DISCUSSION

This study demonstrates that human monocytes not only release substantial amounts of TNF-α after in vitro challenge with the neurokinins SP, NKA and [β-Ala^8]-NKA (4–10), as well as with the tumor promoter PMA, but can also represent a suitable target for the therapeutic effects of cyclosporin A in RA. In fact, CsA effectively inhibits PMA- and tachykinins-induced TNF-α production from human monocytes in vitro. This effect is fully evident at 100 ng/ml, that represents the therapeutic plasma concentration for RA treatment (Cutolo et al., *1998*).

Although CsA has been claimed to be a tachykinin NK_1 receptor antagonist, as it inhibited SP binding in lymphoblastoid cells, human astrocytoma cells and guinea-pig lung parenchima and also reduced SP-evoked IL-6 release from U-373 MG astrocytoma cells (Gitter et al., *1995*), it is not the case in the present study. In fact, inhibition of SP binding was observed at high concentrations (IC_{50} values ranging from 425 to 783 nM; Gitter et al., *1995*), far above the one (100 ng/ml, that is 83 nM) used in this paper. Even more interesting is the fact that one month CsA therapy is able to potently reduce the secretion of TNF-α in monocytes isolated from RA patients: the inhibition amounted to 70% when PMA was the stimulant and to 80–90% in the case of tachykinins. These observations are consistent with those indicating monocytes as key elements in RA, as they present increased adhesiveness, integrin expression and cytokine release (Leirisalo-Repo et al., *1995*; Lioté et al., *1996*) and also agree with our previous data indicating a greater sensitivity (evidenced as increased superoxide anion production and TNF-α mRNA expression) to NK_2 receptor stimulation in monocytes from RA patients (Brunelleschi et al., *1998*). In our opinion, these results indicate that CsA effectiveness in RA not only relies on its ability to inhibit T-lymphocyte activity, but also on its effects on human monocytes.

On the basis of the cytokine secretion pattern, T helper cells are classified in two distinct subsets: Th1 (T helper type 1), producing IL-2, tumor necrosis factor -beta and interferon-gamma, and Th2 (T helper type 2), producing IL-4, IL-5, IL-6 and IL-10 (Rooney et al., *1994*). These subsets differentially promote delayed-type hypersensitivity or antibody responses, respectively. It has been reported that neuropeptides, by direct interaction with T cells, induce cytokine secretion and can break the commitment to a distinct phenotype, SP stimulating cytokine secretion mainly from antigen-activated T-lymphocytes (Levite, *1998*). By inhibiting calcineurin activity, CsA inhibits NF-AT, a transcription factor required for the expression of IL-2 gene in lymphocytes (Liu, *1993*) and so primarily affects Th1 cells (Schreiber and Crabtree, *1992*). However, the NF-AT complex consists of at least two components, one of which – termed NF-ATp-is a common regulatory factor for both Th1 and Th2 cytokine genes (Rooney et al., *1994*). It was recently suggested that the therapeutic efficacy of CsA in RA might relate to its ability in correcting the Th1/Th2 imbalance, as demonstrated by the decrease in IL-2 and TNF-α and the increase in IL-10 serum levels in CsA-treated RA patients (Kim et al., 2000).

Although originally identified as a monocyte factor, TNF-α is now known to be produced also by B- and T-lymphocytes when stimulated by a variety of inducers. The transcription of TNF-α gene is one of the earliest events occurring after stimulation of B- or T-cells via their antigen receptors; CsA blocks TNF-α gene transcription in human B- and T-lymphocytes as well as in monocytes (Andersson et al., *1992*; Boussiotis et al., *1994*; Schmidt et al., *1994*).

On their own, SP and NKA have been demonstrated to induce TNF-α release (Lotz et al., *1988*; this paper) and to enhance TNF-α mRNA expression in circulating human monocytes (Brunelleschi et al., *1998*).

Another interesting link between SP and TNF-α emerges from in vivo data in the mouse. By evaluating the role of tachykinins in LPS- induced production of proinflammatory cytokines, Dickerson et al. (*1998*) demonstrated that animal pretreatment with capsaicin (which destroys C-sensory fibers and blocks tachykinin synthesis) before LPS administration resulted in down-regulation of TNF-α transcription and in reduction of TNF-α release, with no effect on IL-1 and IL-6 secretion. Interestingly, NK_1 receptor antagonists (but not NK_2 ones) reduced TNF-α gene transcription and secretion (Dickerson et al., *1998*).

TNF-α activates at least two cell surface receptors, TNF receptor-1 (TNFR1) and TNF receptor-2 (TNFR2), that are expressed in most cell types (Tartaglia and Goeddel, *1992*). Evidence shows that the intracellular signals that couple TNFR1 include an intracellular signal protein termed 'TNF receptor-associated death domain' (TRADD). Upon engagement of TNFR1, this intracellular signal protein acts as an adapter by recruiting the downstream transducer TRAF2, which stimulates nuclear factor-kappa B (NF-κB) activation (Hsu et al., *1996*).

NF-κB, originally described as a constitutive transcription factor in mature B-cell lines and subsequently found in many other cell types, is present as an inactive complex in the cytoplasm, where it is bound to its inhibitory subunit IκB. NF-κB plays a pivotal role in the development of immune responses and can be activated by a large array of different stimuli, for instance LPS, viruses, inflammatory cytokines, UV light, phorbol esters (see Baeuerle and Henkel, *1994*, for a review).

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Although not evaluated in our study, it seems likely that this transcription factor might represent a common link among SP, TNF-α and CsA, since all these agents have signal transduction pathways involving NF-κB.

While TNF-α is one of the most potent activator of this transcription factor (Baeuerle and Henkel, 1994; Wallach, 1997), CsA has been shown to negatively interfere with NF-κB (Marienfeld et al., 1997), besides inhibiting NF-AT activity.

Interestingly, CsA reduces LPS-induced expression of tissue factor (TF) in monocyte/macrophages and, by decreasing the nuclear translocation of NF-κB, also inhibits LPS-induced activation of NF-κB (Holschermann et al., 1996). CsA has also been demonstrated to prevent the PMA-mediated down-regulation of IκB alpha in Jurkat T cells (Lai and Tan, 1994).

In U373 MG astrocytoma cells, low concentrations of SP ($10^{-10} - 10^{-6}$M) potently triggered activation of NF-κB (Lieb et al., 1997). SP-induced NF-κB activation, which was completely prevented by a NK_1 selective antagonist, was associated with the enhanced mRNA expression and secretion of IL-8 (which represents an NF-κB-controlled target gene). In this cellular system, CsA dose-dependently reduced SP-triggered NF-κB activation as well as SP-evoked IL-8 release (Lieb et al., 1997). More recently, SP treatment of human dermal microvascular endothelial cells has been shown to coincidently activate NF-κB and NF-AT transcription factors, CsA reducing both responses (Quinlan et al., 1999).

In conclusion, we have demonstrated that monocytes isolated from RA patients, when challenged in vitro with phorbol esters or tachykinins, secrete greater amounts of TNF-α as compared to controls, and that CsA, either in vitro than ex-vivo, potently inhibits TNF-α release. This demonstrated inhibitory effects participate in the therapeutic activity of CsA and may contribute to its clinical efficacy in RA.

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