Taylor & Francis healthsciences

Mediators of Inflammation, 11, 53–59 (2002)

Immunomodulating effects of tofizopam (Grandaxin®) and diazepam *in vitro*

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BENZODIAZEPINES (BDZs) are known to act not only in the central nervous system, but on peripheral cells and tissues binding to the peripheral-type benzodiazepine receptors. In the present study, the influence of two different BDZs (diazepam (Dz) and tofizopam (Tof)) on several immune functions has been examined ***in vitro***. Some differences between Dz and Tof in their effects on human lymphocyte proliferative response, changes in glucocorticoid-induced suppression of cell proliferation and influence on cytokine production (tumor necrosis factor-α (TNF-α) and interleukin-2 (IL-2)) have been determined. Dz suppressed mitogen-induced peripheral blood mononuclear cell (PBMC) proliferation, enhanced dexamethasone-induced inhibition of PBMC proliferative response, and suppressed lymphocyte production of TNF-α and IL-2. Tof usually enhanced PBMC proliferation and IL-2 production in low and moderate doses, but in high doses it suppressed both. Tof in all investigated doses enhanced dexamethasone-induced suppression of lymphocyte proliferation and depressed TNF-α production. Thus, both Dz and Tof are shown to have immunomodulating effects ***in vitro.*** Tof, opposite to Dz even in the therapeutic doses, is able to enhance ***in vitro*** mitogen-induced lymphocyte proliferation and IL-2 production.

Key words: Benzodiazepines, Tofizopam, Diazepam, Immunomodulating effects, Human peripheral blood mononuclear cells, Dexamethasone susceptibility, TNF-α, IL-2

Introduction

During the past two decades, it has become clear that the immune system responds to a great amount of factors originating in the endocrine, central and autonomic nervous systems. Recently, it has become clear that there is also a feedback loop from the immune system to the other systems. (The reverse direction of influence also exists.) The regulation of neuroendocrine function is highly sensitive to the wide array of cytokines released from activated immune cells.[1,2] The bi-directional communication between these systems is based on the expression of mediators, receptors and use of signal transduction mechanisms that are common to both immune and endocrine cells and to neurons and glia in the central nervous system (CNS).[2,3]

One of the important links in the neuro-immunoendocrine relations are the central and peripheral benzodiazepine receptors (BDZ-R), which together with their ligands form the molecular basis of a novel regulatory network that contributes to the effects of anxiety on immune status.[4] Benzodiazepines (BDZs) are frequently prescribed because of their anxiolytic, anticonvulsant, muscle-relaxant and hypnotic properties. Many articles reviewed the important topic of the complex relations between BDZs, the stress axes (hypothalamo-pituitary-adrenal and autonomic) and the immune system.[4–6]

Two types of BDZ-R have been identified. The first, namely central BDZ receptors (CBR), present exclusively in the CNS and are localized to neurons.[7] BDZs bind to a domain that regulates chloride flux by modulating α-aminobutyric acid (GABA) binding to $GABA_A$ receptors.[8] The second class is the peripheral-type BDZ receptors (PBR) that are ubiquitously distributed in peripheral tissues[9] as well as in glia of the CNS.[10] Our understanding of the PBR is still far from complete. They participate in cholesterol transport, heme biosynthesis, proliferation of different cell lines and immunomodulation.[5] It has been suggested now that the PBR may serve as a receptor in immune cells, which can be a critical locus in the inter-relation between brain, behavior and immunity.[11]

The BDZs diazepam (Dz)[9] and tofizopam (Tof)[12,13] are related to a mixed type of BDZs that interact both with CBR and PBR by different mechanisms.[12,13] In man, Tof (a 2,3-benzodiazepine) has been shown to have anxiolytic activity but, in contrast to the widely

ISSN 0962-9351 print/ISSN 1466-1861 online/02/010053-07 © 2002 Taylor & Francis Ltd
DOI: 10.1080/09629350120117301

used 1,4-benzodiazepines (Dz), it has no appreciable sedative and muscle relaxant effects.[12,14]

In the present study, some differences between Dz and Tof in their effects on several immune functions *in vitro* are described.

Material and methods

Cell cultures

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized peripheral blood of healthy volunteers by Ficoll-Verographin gradient centrifugation. The cells were washed twice and resuspended in RPMI-1640 medium (ICN, Irvin, CA, USA) supplemented with 2×10^{-3} M HEPES, 2 mM L-glutamine, 2.8×10^{-6} M 2-mercaptoethanol, 20 μg/ml of gentamycin and different serums. Ten percent of heat-inactivated fetal calf serum (FCS) (ICN) were used for PBMC proliferation, tumor necrosis factor-α (TNF-α) production and interleukin-2 (IL-2) assay, and 1% of FCS used for IL-2 production.

Lymphocyte proliferation

PBMC were cultivated in flat-bottomed 96-well plates (Nunc, Roskild, Denmark), and contained 5×10^4 cells in each well. The final concentration of phytohemagglutinin (PHA) (Calbiochem, La Jolla, CA, USA) was 5 μg/ml. Modulation of lymphocyte proliferation by Tof and Dz was evaluated at four different concentrations within the dose ranges of 0.4–50 μg/ml for Tof and of 0.04–5 μg/ml for Dz. The control wells incubated without BDZs contained a culture medium with mitogen or a culture medium only. The influence of Tof and Dz on the inhibition of PHA stimulation by dexamethasone (Dx) (Sigma, St. Louis, MO, USA) was also evaluated. Dx was used at the concentrations of 10^{-8} and 10^{-7} M with or without (control wells) the above doses of Tof and Dz. The cells were incubated for 72 h at 37°C in a humidified atmosphere (95% air, 5% CO_2). Four hours before the end of cultivation, each well was pulsed with 40 kBq of [^{3}H]-thymidine (Isotope, Moscow, Russia). The cells were harvested with a cell harvester and counted on a liquid scintillation counter. Four wells of each concentration were assayed and the counts per minute (count/min) were averaged.

TNF-α production

Cells (10^6 cells/ml) were cultivated for 16 h at 37°C with 5 μg/ml of PHA and BDZs at three different concentrations (7.5, 15 and 30 μg/ml for Tof, and 1.25, 2.5 and 5 μg/ml for Dz) in a humidified atmosphere containing 5% CO_2 in the wells (1.5 ml per well) of 24-well plates (Nunc). The cells in the control wells were incubated with PHA only. The supernatants were collected and stored at –20°C until cytokine activity examination.

IL-2 production

PBMC in the concentration of 10^6 cells/ml were resuspended in the culture medium with 20 μg/ml of concanavalin A (Calbiochem). The cells were incubated (see earlier) for 16 h in the wells (1.5 ml per well) of 24-well plates (Nunc) with the addition of BDZs at three different concentrations (7.5, 15 and 30 μg/ml for Tof, and 1.25, 2.5 and 5 μg/ml for Dz). The cells in the control wells were incubated with concanavalin A only. The supernatants were collected and stored at –20°C until use.

TNF activity assay

TNF activity was determined by the method of Ruff and Gifford[15] with some modifications. Briefly, L929 cells were seeded at a density of 3×10^4 cells per well in 96-well plates in 100 μl of medium 199 to which 10% heat-inactivated calf bovine serum and gentamycin had been added. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 until the monolayer formation. After the culture medium elimination, twofold serial dilution of the samples (100 μl of each dilution) and 100 μl of fresh culture medium with 20 μg/ml of actinomycin D (Serva, Heidelberg, Germany) were added, and further incubated for 18 h under the same conditions. Supernatants were then removed and cells stained with 0.2% crystal violet (Sigma). After washing and drying, plates were finally read at 594 nm on a Titertek Multiskan microElisa reader. Human recombinant TNF-α (Institute of Bioorganic Chemistry, Moscow, Russia) was used as internal standard. For the comparison of experimental and calibrating curves, a probit-analysis method was used.[16] The TNF content in the samples was expressed in picograms per milliliter.

IL-2 activity assay

The amount of IL-2 in separate samples was determined by their ability to maintain the growth of the IL-2-dependent cytotoxic T-cell line (CTLL-2). Supernatants were twofold serially diluted and 100 μl of samples from each dilution and 100 μl of CTLL cell suspension (4×10^3 cells per well) were added to triplicate wells of a 96-well flat-bottomed plate. Triplicate cell control wells received 100 μl of culture medium instead of samples. Recombinant human IL-2 (code No 86/564; National Institute for Biological Standards and Control, Hertfordshire, UK) and IL-2 containing preparation Ronkoleukin (Cytokine, St. Petersburg, Russia) were used as standards. After 20 h incubation at 37°C and 5% CO_2, the colorimetric MTT assay was carried out[17] with some modifications.

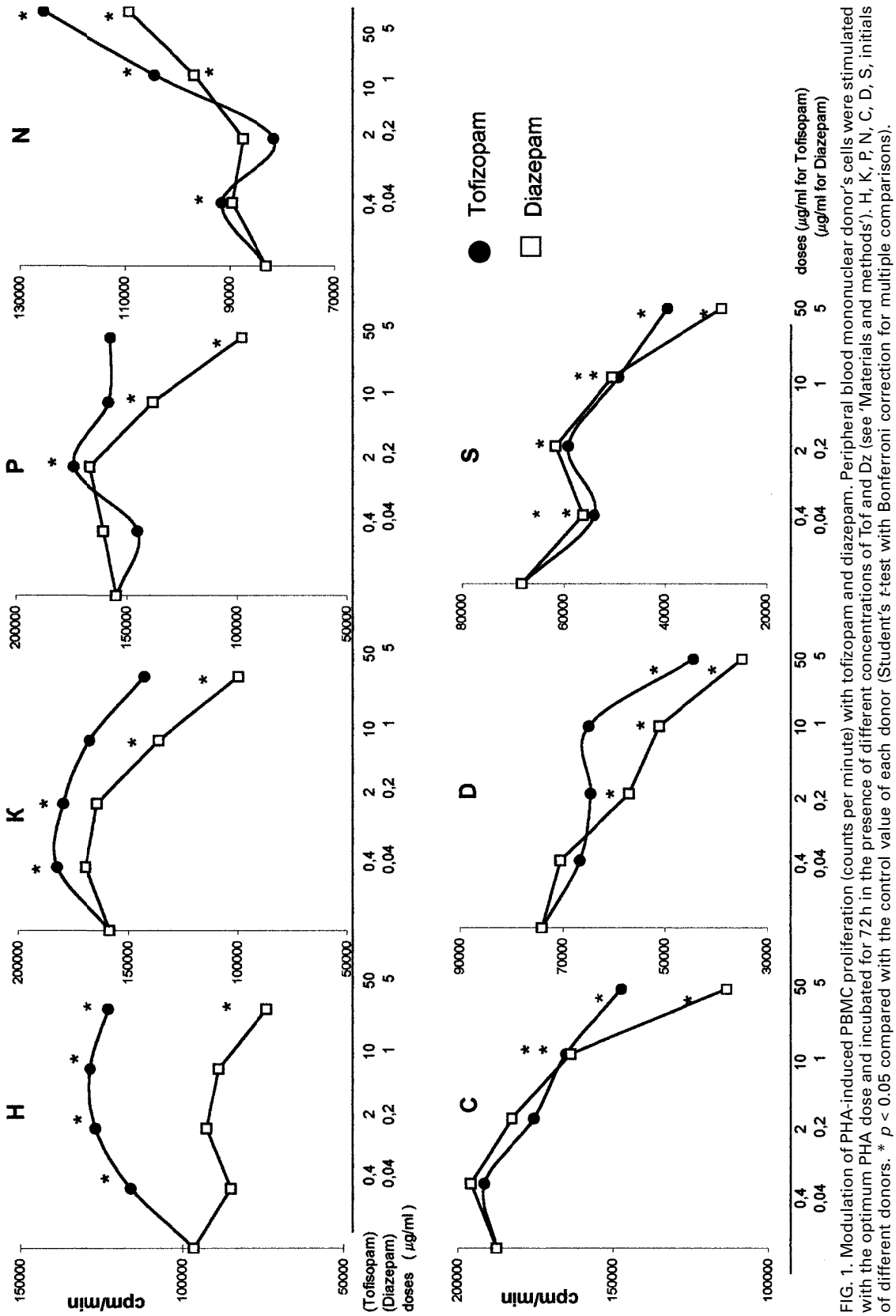

FIG. 1. Modulation of PHA-induced PBMC proliferation (counts per minute) with tofizopam and diazepam. Peripheral blood mononuclear donor's cells were stimulated with the optimum PHA dose and incubated for 72 h in the presence of different concentrations of Tof and Dz (see 'Materials and methods'). H, K, P, N, C, D, S, initials of different donors. * $p < 0.05$ compared with the control value of each donor (Student's *t*-test with Bonferroni correction for multiple comparisons).

Table 1. Changes in dexamethasone (Dx)-mediated inhibition of phytohemagglutinin (PHA)-induced lymphocyte proliferation caused by different doses of tofizopam (Tof) and diazepam (Dz)

Dx dose (nM)	Tof/Dz	Dose (μg/ml)	Donor K	Donor P	Donor C	Donor H	Donor D
10	Tofizopam	–	123 ± 3.4	56 ± 9.1	49 ± 6.6	34 ± 2.5	24 ± 1.3
		0.4	103 ± 7.9	48 ± 11.9	49 ± 5.6	30 ± 1.2	23 ± 1.9
		2	85 ± 3.7*	54 ± 5.5	37 ± 0.7	30 ± 1.4	22 ± 1.1
		10	85 ± 8.3*	42 ± 6.7	35 ± 4.6	29 ± 1.7	21 ± 0.5
		50	74 ± 4.9*	42 ± 2.9	31 ± 2.5	25 ± 1.7	13 ± 1.7*
100	Tofizopam	–	31 ± 2.2	32 ± 4.7	5.4 ± 0.7	17 ± 1.7	11 ± 0.4
		0.4	23 ± 2.3	27 ± 4.3	5.3 ± 1.3	15 ± 1.9	11 ± 1.6
		2	26 ± 4.8	22 ± 4.3	3.9 ± 0.8	7.2 ± 0.9*	8.4 ± 1.2
		10	23 ± 1.3*	21 ± 2.5	4.1 ± 0.1	7.9 ± 0.5*	8.7 ± 0.4*
		50	15 ± 2.6*	15 ± 4.4*	2.9 ± 0.3*	6.6 ± 0.8*	7.3 ± 1.6
10	Diazepam	–	123 ± 7.7	56 ± 9.1	49 ± 6.6	34 ± 2.5	24 ± 1.3
		0.04	121 ± 3.6	90 ± 5.7*	40 ± 9.2	38 ± 2.7	30 ± 2.2
		0.2	91 ± 1.8*	70 ± 8.9	35 ± 3.7	30 ± 2.7	28 ± 1.9
		1	74 ± 4.1*	48 ± 3.0	26 ± 0.7*	25 ± 2.2	18 ± 1.1*
		5	24 ± 3.3*	29 ± 6.0*	17 ± 2.9*	11 ± 0.4*	4.6 ± 0.6*
100	Diazepam	–	31 ± 2.2	32 ± 4.7	5.4 ± 0.7	17 ± 1.7	11 ± 0.4
		0.04	31 ± 8.6	29 ± 5.5	5.2 ± 0.9	18 ± 4.1	11 ± 1.3
		0.2	21 ± 2.6*	26 ± 2.6	4.6 ± 0.7	8.4 ± 0.8*	11 ± 0.2
		1	15 ± 1.2*	21 ± 3.3	3.6 ± 0.8	8.1 ± 1.9*	6.0 ± 0.7*
		5	4.8 ± 1.1*	5.7 ± 1.1*	2.2 ± 0.9*	2.5 ± 0.6*	2.4 ± 3.3*

PHA-stimulated donor's peripheral blood mononuclear cells (PBMC) were cultivated (see 'Materials and methods') with 10 or 100 nM of Dx and with or without different doses of Tof and Dz.
Data are presented as counts per min × 1000 and are expressed as mean ± standard error of the mean. Donors K, P, C, H, and D correspond to healthy volunteers.
*$p < 0.05$; in comparison with mean proliferative response only with Dx, without BDZs (–); Student's *t*-test with Bonferroni correction for multiple comparisons. Proliferation of the same donor's PBMC without Dx can be seen in Fig. 1.

Briefly, 20 μl of 5 mg/ml MTT (Sigma) stock solution were added to each well. Plates were incubated again at the same conditions for 4 h. The supernatants were then removed and 100 μl of dimethylsulfoxide (Sigma) were added to all wells to dissolve the crystals. The plates were read at 570 nm on a Titertek Multiskan microElisa reader. The titration curves of the tested samples and the controls were analyzed by probit analysis.[16] The content of IL-2 was expressed in international units per milliliter.

Statistical analysis

Statistical analysis was performed using the Student's *t*-test with Bonferroni correction for multiple comparisons.

Results

PBMC proliferation in the presence of Dz and Tof

Dz in a high dose (1–5 μg/ml), to a considerable extent, suppressed cell proliferation (Fig. 1) and, as a rule, had no effect in a low dose (0.04–0.2 μg/ml). The enhancing effect of Dz on PBMC proliferation was observed only with lymphocytes of donor N. But in this very case, the higher doses of Dz and Tof caused the greater increase in PHA-induced lymphocyte proliferation.

Tof usually enhanced cell proliferation (Fig. 1, donors H, K, P and N). Only in high doses (10 or 50 μg/ml) and only in three cases from seven (donors C, D and S) did Tof significantly suppress PBMC blast transformation. It should be noted that in three cases the curves of the both BDZs had a nearly complete coincidence (donors N, C and S), but the profile of the other curves was different (donors H, K, P and D). Thus, Tof could reliably enhance PBMC proliferation in low and moderate doses, whereas Dz caused significant suppression in doses of 1 and 5 μg/ml (or only 5 μg/ml) and had no effect in lower doses.

Changes in Dx-mediated inhibition of PHA-induced lymphocyte proliferation

Changes in lymphocyte susceptibility to glucocorticoid hormones caused by Tof and Dz are presented in Table 1. Dose-dependent enhancing of Dx-mediated inhibition of PHA-induced lymphocyte proliferation was observed as for Tof and for Dz at two corticosteroid concentrations (10 and 100 nM). It is necessary to mention that, with donors H, K and P, lymphocytes tofizopam itself caused the rise of lymphocyte proliferation (Fig. 1) but, in the presence of Dx, Tof increased its suppression. Significant suppression of lymphocyte proliferation in comparison with proliferative response only with Dx without BDZs was shown for high and average doses of Tof and Dz.

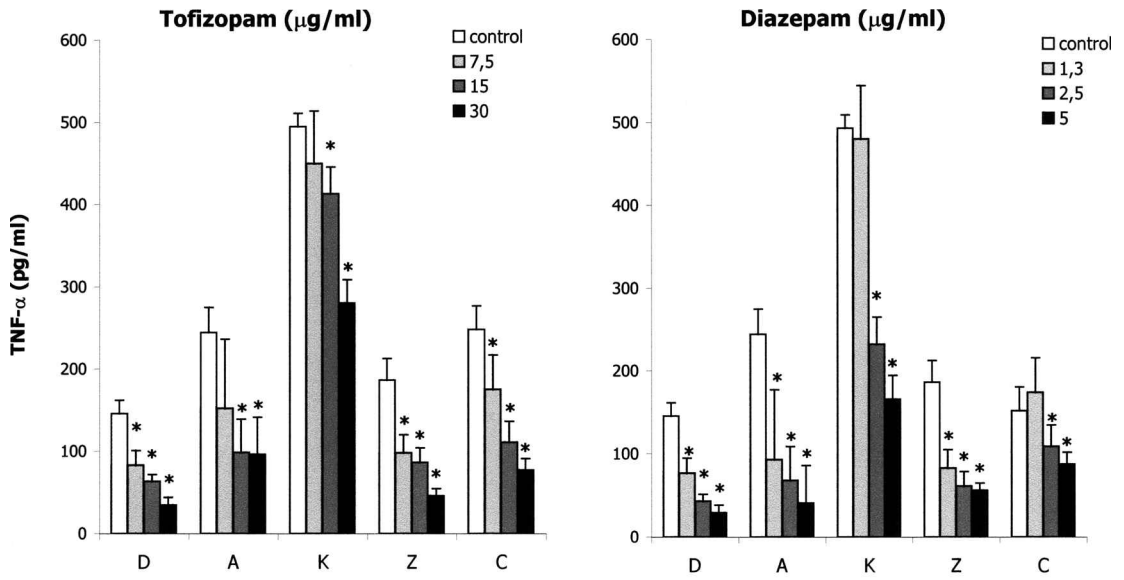

FIG. 2. The effect of tofizopam and diazepam on *in vitro* TNF-α production by PBMC of five healthy donors. The supernatants were obtained and collected, and the TNF-α activity was quantified as described in 'Materials and methods'. D, A, K, Z, C, initials of the healthy volunteers. * $p < 0.05$ compared with the control value of each donor (Student's *t*-test with Bonferroni correction for multiple comparisons).

Enhancing of Dx-induced suppression by BDZs did not depend on individual susceptibility to glucocorticoids.

Changes in TNF production

In the experiments of cytokine production, we used not so wide but therapeutically used range of doses of BDZs. We examined the influence of Tof and Dz on TNF-α production by PHA-stimulated PBMC of five healthy volunteers. Neither Tof nor Dz in analyzed doses affected the growth of the L929 test-cell line. Dose-dependent suppression of TNF-α production has been shown for both of the BDZs (Fig. 2) ($p < 0.05$).

Changes in IL-2 production

The influence of Tof (7.5, 15 and 30 μg/ml) and Dz (1.25, 2.5 and 5 μg/ml) on IL-2 production was examined on lymphocytes of three healthy volun-

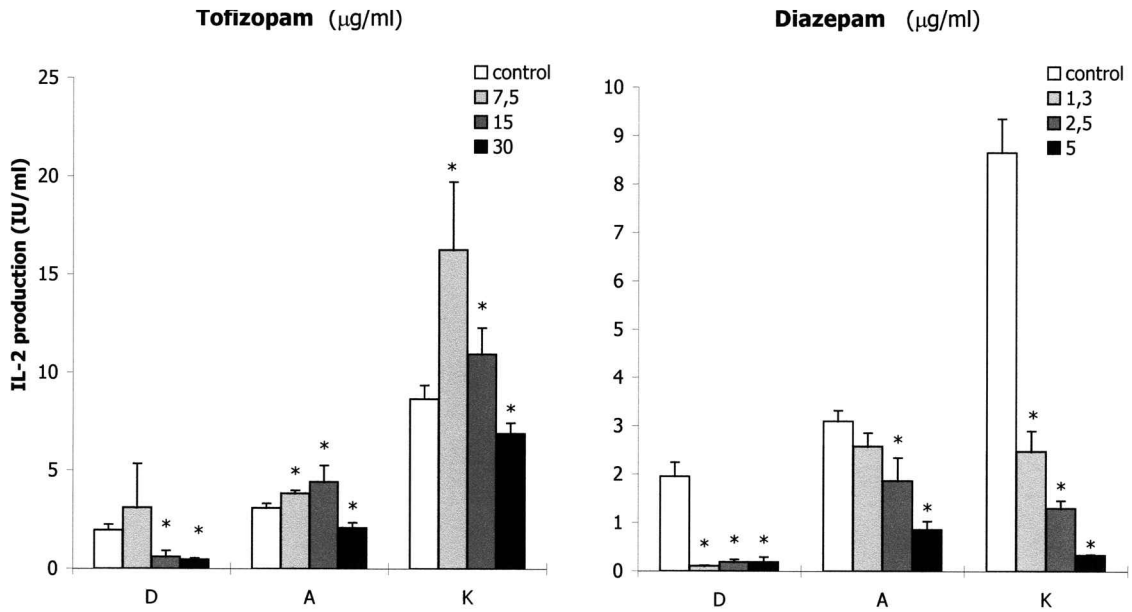

FIG. 3. The effect of tofizopam and diazepam on *in vitro* IL-2 production by PBMC of three healthy donors. The supernatants were obtained and collected, and the IL-2 activity was quantified as described in 'Materials and methods'. D, A, K, initials of healthy volunteers. * $p < 0.05$ compared with the control value of each donor (Student's *t*-test with Bonferroni correction for multiple comparisons).

teers. The enhancing effect on IL-2 release treated with Tof was marked with PBMC from all of the volunteers (Fig. 3). Not all of the doses gave an expressed effect. The doses of 7.5 and 15 μg/ml significantly stimulated production of this cytokine for two donors (Fig. 3, donors A and K; $p < 0.05$). The dose of 7.5 μg/ml insignificantly enhanced and the dose 15 of μg/ml significantly suppressed IL-2 release from lymphocytes of donor D. The dose of 30 μg/ml (maximal therapeutic concentration in serum) significantly suppressed IL-2 production for all donors ($p < 0.05$). Our results indicate that the effect of Tof on IL-2 production could vary from marked enhancement to appreciable suppression. Thus, we notice a dose-dependent bell-shaped curve. Also, the same dose could elicit different effects for different individuals. Dz as opposed to Tof suppressed IL-2 production in all investigated doses ($p < 0.05$), but also in this case the identical doses cause various degrees of effects for different individuals.

It should be mentioned that only high doses (> 50 μg/ml for Tof and > 10 μg/ml for Dz) of BDZs, which were not used in the experiments, slightly reduced CTLL proliferation.

Discussion

The presented results indicate that the mixed-type BDZs diazepam and tofizopam are able to modulate the immune responses *in vitro*: lymphocyte proliferation response management, change in glucocorticoid susceptibility and influence on cytokine production (TNF-α, IL-2). The former behaves as an immunosuppressive agent in therapeutic doses, whereas the latter seems to upregulate immune functions (PBMC blast transformation, IL-2 production) in some conditions.

This modulation *in vitro* is realized not through CBR but through PBR, at least partially. PBR have been identified on thymocytes,[18] macrophages,[19] neutrophils,[20] leukocytes[21] and lymphocytes.[22] The PBR is implicated in both stimulation of cell growth rate and DNA synthesis and in inhibition of cell proliferation.[23,24] The former requires nanomolar concentrations, whereas the latter requires micromolar concentrations of PBR ligands. This ambiguity of the PBR in the mediation of benzodiazepine-induced effects on mitosis has been suggested to be due to the existence of receptor subtypes[25] or another BDZ low affinity binding site (platelet-activating factor receptor[26]). The immunomodulatory action of benzodiazepines may be of pharmacological importance because, during their application, their plasma concentrations reach micromolar levels.

It is known that chronically treated BDZ users had some alterations in immune and neuroendocrine system.[27] Hence it is necessary to emphasize meaning of the received data about an opportunity of BDZs to increase corticosteroid-induced (stress-induced) immunosuppression. It is more interesting to search among the novel BDZs,[28] those with immunostimulatory activity. Tof as opposed to Dz, even in the therapeutic doses, is able to enhance *in vitro* mitogen-induced lymphocyte proliferation and IL-2 production.

Each person has an individual susceptibility to mitogen or antigen-induced lymphocyte stimulation, glucocorticoids and BDZs. That is why a certain type of BDZ should be administered in individually selected doses and courses. They should be prescribed with caution, especially to immunocompromised patients (e.g. because of aging, treatment with immunosuppressive drugs, suffered from chronic obstructive pulmonary disease (COPD), chronic heart or renal failures, etc.).

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Received 17 October 2001
Accepted 3 December 2001