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ORIGINAL RESEARCH

Long-Term Effects of Amino-Bisphosphonates on Circulating $\gamma\delta$ T Cells

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Abstract The aim of this study was to explore whether desensitization to the occurrence of the acute-phase response (APR) in patients previously treated with amino-bisphosphonates (N-BPs) is due to a long-lasting reduction in the number of circulating $\gamma\delta$ T cells. Circulating lymphocyte subpopulation counts were obtained from 63 patients with postmenopausal or senile osteoporosis at baseline and after 2 days and 12 months of the first intravenous (IV) 5 mg zoledronic acid (ZOL) infusion. At baseline both the proportion and absolute number of circulating $\gamma\delta$ T cells were significantly higher in patients who had never used N-BPs vs. previous users, either oral or IV. A typical APR was observed in none of the patients given IV ZOL a year earlier, in 6 (22 %) of the patients previously treated with oral N-BPs, and in 13 (57 %) of the patients naive to any N-BP treatment. In patients naive to N-BPs, a significant reduction in both total lymphocytes and their subsets was observed 2 days after ZOL infusion; all these changes returned to baseline values 1 year later with the exception of $\gamma\delta$ T cells, which remained significantly lower in terms of both proportion and absolute number. These results indicate for the first time that both IV and oral N-BP treatments are associated with a long-lasting decrease in circulating $\gamma\delta$ T cells, and this may explain the lower incidence of APR in patients previously exposed to N-BPs. Other clinical implications of this sustained effect of N-BPs on immune-regulatory cells might be important.

Keywords T cell · $\gamma\delta$ T cell · Bisphosphonate · Acute-phase response

The authors have stated that they have no conflict of interest.

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Introduction

Amino-bisphosphonates (N-BPs) are now established therapies for osteoporosis and Paget disease, and they are widely used for the prevention and treatment of skeletally related events in cancer. The use of intravenous (IV) N-BPs is occasionally associated with the appearance within 24–36 hours of fever and musculoskeletal pain [1]. A fall in circulating lymphocyte numbers [1, 2] and increases in serum IL-6 [2, 3] and TNFα [3] also have been reported. This is referred to as the acute-phase response (APR). N-BPs inhibit osteoclastic bone resorption by blocking farnesyl-pyrophosphate-synthase, an enzyme in the mevalonate pathway. Recent work has suggested that this action may underlie development of the APR since intermediates in this pathway, isopentenyl diphosphate and dimethylallyl diphosphate, accumulate in monocytes when this enzyme is blocked and this results in the activation of adjacent γδ T cells with the release of interferon-γ and TNFα [4–8]. γδ T cells are nonconventional T cells that, unlike conventional αβ T cells, can recognize antigen without the need for presentation by MHC-class molecules; in humans, γδ T cells comprise only a minor proportion (1–10 %) of $CD3^+$ T cells in peripheral blood. In addition to antibacterial effects, γδ T cells seem to play an important role in tumor surveillance [9].

The APR is more common in younger subjects, nonsteroidal anti-inflammatory drug users, and those having back pain and less common in smokers, diabetics, and calcitonin or previous bisphosphonate users [10]. The APR is considerably more common after the first infusion and tends to disappear despite continuing therapy, and both incidence and severity decrease substantially with subsequent treatments [1, 10]. The reason for this desensitization is poorly understood. Recently, we observed that the proportion of circulating γδ T cells is an important determinant of the occurrence of the APR after IV infusion of zoledronic acid (ZOL) and possibly of any other N-BP [11]. The aim of this study was to explore whether desensitization to the occurrence of the APR in patients previously treated with N-BPs might be due to a long-lasting reduction of circulating γδ T cells.

Materials and Methods

Patients

Sixty-eight patients with postmenopausal or senile osteoporosis, 5 men and 63 women with a mean age of 74 years (range 45–91, SD 9 years) were enrolled in this study. Patients with cancer, autoimmune diseases, immunodeficiency, severe liver or renal insufficiency (serum creatinine >1.0 mg/dL), or recent acute infections were excluded from this study. Patients were not eligible if they had been treated within the last 2 years with cytostatic drugs, corticosteroids, or immune therapeutics. Thus, three patients were excluded for the presence of incidental inflammatory processes and two for a recent treatment course with corticosteroids. Twenty-three of the recruited patients never received either oral or IV N-BP treatment, 25 patients had been on treatment with oral N-BPs (20 with alendronate and five with risedronate) for 10–48 months (mean 18 ± 14 SD) up to 1–6 months before, and 15 patients received IV ZOL 1 year earlier.

Treatment and Follow-up Investigation

All study participants received a single dose of 5 mg ZOL in 100 mL of 0.9 % saline IV infusion over 15 min. Before the IV infusion all patients had been on vitamin D supplements for at least 2 months.

Immediately before ZOL infusion, samples of peripheral blood were taken in fasting conditions in the morning using Vacutainer blood collection tubes coated with ethylenediamine-tetraacetic acid. Similar samples were taken also after 2 days and 1 year in the 48 patients who had never received IV N-BP treatment.

White blood cells (WBCs) were counted with an automated hematology analyzer (ADVIA 2120i; Siemens, Malvern, PA). Fifty microliters of blood were distributed into each tube by the automated BD FACS Sample Prep Assistant II (Becton Dickinson, Mountain View, CA), a mixture of monoclonal antibodies conjugated with different fluorochromes (FITC, PE, PerCP, PE-Cy7, APC, APC-Cy7; BD Biosciences, San Diego, CA) was added, the red blood cells were lysed, and finally the cells were fixed (BD FACS Lysing Solution). Lymphocytes were analyzed by flow cytometry (BD FACSCanto, Becton Dickinson) with BD FACS Diva software. Lymphocytes were isolated using CD45 versus SSC as a gating strategy. Different subsets of T cells were counted using these monoclonal antibodies: APC-conjugated anti-CD3, FITC-conjugated anti-CD4, PE-Cy7-conjugated anti-CD8. γδ T cells were counted in the samples of $CD3^+$ T lymphocytes stained with anti-TCR γ/δ-PE. The laboratory used UK NEQAS (www.ukneqas.org.uk) for leukocyte immunophenotyping to ensure external quality.

Body temperature was determined with digital clinical thermometers immediately before the IV infusion and at 12-hour intervals for 3 days. Fever was defined as an increase in body temperature above 37.0 °C. Patients were instructed to register the temperature values in a diary together with any self-administered acetaminophen dose to treat fever or other symptoms of APR.

Peripheral leukocyte and lymphocyte subpopulations were compared in patients with and without previous use of

N-BPs, by the Mann–Whitney *U*-test for nonparametric independent variables and then, after correcting the values for any potential interfering factor, by ANCOVA. In order to detect a 25 % difference in $\gamma\delta$ T cells, at least 15 patients per group were required for a 5 % alfa error and statistical power >90 %. A two-tailed *p* value of 0.05 was considered significant. SPSS software (version 17.00; SPSS, Inc., Chicago, IL) was used for statistical analysis.

This study was approved by the local ethics committee, and the subjects' consent was obtained according to the Declaration of Helsinki.

Results

At baseline no significant differences were observed between never or previous N-BP users for age, WBCs, lymphocytes, or T cells ($CD3^+$) (Table 1). Both proportion and absolute number of circulating $\gamma\delta$ T cells were significantly higher in patients who had never used N-BPs vs. previous users, either oral or IV (Table 1). The number of $\gamma\delta$ T cells was 44 ± 24/μL in the 23 patients naive to N-BP treatment, 28 ± 16/μL in the 25 patients previously treated with oral N-BPs ($p = 0.011$ vs. never treated), and 25 ± 19/μL in the 15 patients who had received ZOL 5 mg IV 1 year earlier ($p = 0.014$ vs. never treated). The differences in circulating $\gamma\delta$ T cells (both absolute number and percentage) remained unchanged and equally significant (same *p* values) for values adjusted for age, another important determinant of circulating $\gamma\delta$ T cells [11–13]. The significance of the observation was maintained when corrected for multiple comparisons (Bonferroni test).

All patients were given IV 5 mg ZOL. A typical APR was observed in none of the patients given IV ZOL a year earlier, in 6 (22 %) of the patients previously treated with oral N-BPs, and in 13 (57 %) of the patients naive to any N-BP treatment ($p < 0.01$). The baseline values of WBCs, lymphocytes, and their subsets in patients with and without APR are listed in Table 2, both the proportion and absolute number of $\gamma\delta$ T cells were significantly higher in patients who experienced an APR.

In all patients, a transient increase in total WBCs, mainly due to granulocyte count (data not shown), was observed. In patients naive to N-BPs this was associated with a significant reduction in both total lymphocytes and their subsets (with exclusion of NK) 2 days after ZOL infusion (Fig. 1). All these changes returned to baseline values 1 year later with the exception of $\gamma\delta$ T cells, which remained significantly lower in terms of both proportion

Table 2 Baseline counts (cells per microliter or %) of white blood cells, lymphocytes, and their subsets in patients with or without APR

	APR	Mean	SD	*p* between groups
White blood cells	No	5,892	1,542	NS
	Yes	6,426	2,153	
Lymphocytes	No	1,727	452	NS
	Yes	1,726	507	
T cells ($CD3^+$)	No	1,227	340	NS
	Yes	1,244	444	
$CD4^+$ T cells	No	873	280	NS
	Yes	859	373	
$CD8^+$ T cells	No	353	167	NS
	Yes	363	151	
$\gamma\delta$ T cells (%)	No	2.3	1.5	0.002
	Yes	4.0	2.5	
$\gamma\delta$ T cells	No	28	18	0.004
	Yes	45	25	
B cells[a] ($CD19^+$)	No	219	136	NS
	Yes	204	96	
NK cells	No	283	122	NS
	Yes	265	125	

NS nonsignificant ($p > 0.05$)

[a] Cells per microliter

Table 1 Main characteristics (mean ± SD) of the three groups of patients, without or with previous oral or IV N-BPs

	Age (year)	White blood cells[a]	Lymphocytes[a]	T cells ($CD3^+$)[a]	$\gamma\delta$ T cells (%)	$\gamma\delta$ T cells[a]
N-BP-naive ($n = 23$)	72 ± 10	6,282 ± 2,217	1,681 ± 500	1,218 ± 437	3.9 ± 2.3	44 ± 24
Previous N-BPs						
All ($n = 40$)	75 ± 7	5,921 ± 1,427	1,754 ± 448	1,240 ± 333	2.2 ± 1.5	27 ± 17
Oral ($n = 25$)	73 ± 7	6,202 ± 1,590	1,788 ± 502	1,268 ± 366	2.3 ± 1.4	28 ± 16
IV ($n = 15$)	77 ± 8	5,453 ± 981	1,695 ± 348	1,192 ± 275	2.1 ± 1.6	25 ± 19
p N-BP-naive vs. all	NS	NS	NS	NS	0.001	0.002
p N-BP-naive vs. oral	NS	NS	NS	NS	0.006	0.010
p N-BP-naive vs. IV	NS	NS	NS	NS	0.013	0.014

NS nonsignificant ($p > 0.05$)

[a] Cells per microliter

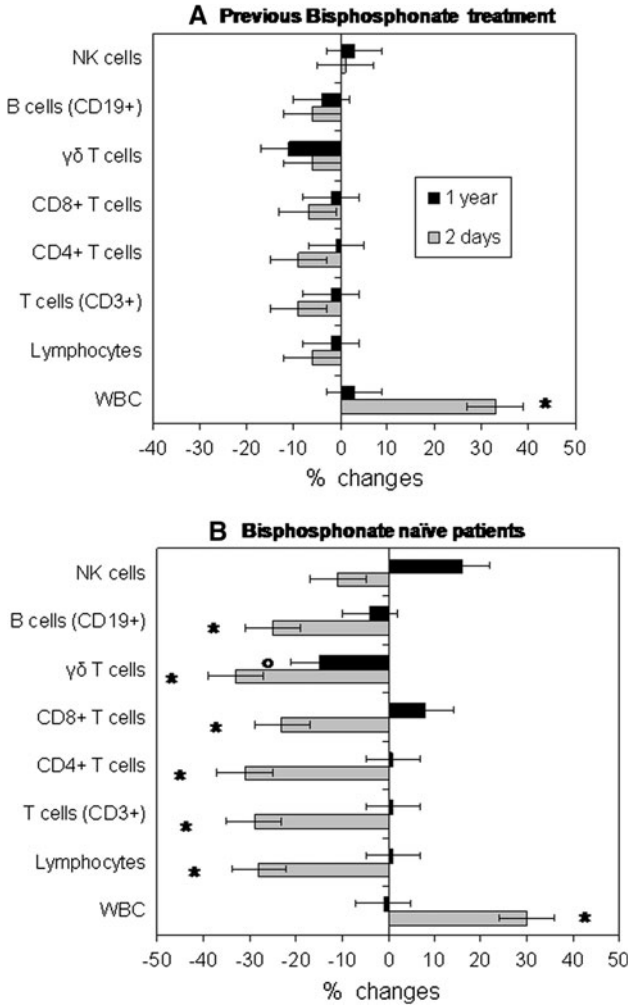

Fig. 1 Mean percentage changes of white blood cells, lymphocytes, and their subset counts 2 days or 1 year after a single IV infusion of zoledronic acid in patients previously treated with oral N-BPs (**a**) or naive to N-BPs (**b**). $*p < 0.01$, $°p < 0.05$ versus baseline

and absolute number (Fig. 1). The changes in total lymphocytes and their subpopulations were greater in the 13 naive patients reporting an APR. In these patients γδ T cells decreased by 51 and 29 % after 2 days and 1 year, respectively (results not shown).

With the limitation that only a few men were included in this study, no significant differences between genders in terms of cell counts was observed in response to N-BP treatment.

Discussion

With this study we have shown that the number and proportion of circulating γδ T cells are significantly lower in patients previously treated with either oral or IV N-BPs compared with N-BP-naive osteoporotic patients and that the first IV ZOL infusion is associated with a rapid decrease in circulating γδ T cells, which persists for at least 1 year.

The evaluation of the acute changes in circulating γδ T cells associated with IV N-BP treatment has yielded so far contradictory results. In the first in vivo evidence on the γδ T cell role as a mediator of the APR, Kunzmann et al. [7] reported an increase in the number of circulating γδ T cells in N-BP (pamidronate)-treated multiple myeloma patients up to 28 days postinfusion. Similarly, ZOL in combination with IL-2 administration has been reported to result in sustained increases in circulating γδ T cells in patients with hormone-refractory prostate cancer [14] and lymphoid malignancies [15]. In two more recent clinical studies in postmenopausal women focused on the efficacy of statin treatment to prevent APR in patients given IV N-BPs [16, 17] a decrease in circulating γδ T cells was observed; but the changes were not significant, and the authors did not discuss this observation. Altogether these results are somewhat suggestive of differences in γδ T cell reactivity between cancer and noncancer patients.

The decrease in circulating levels of γδ T cells we observed might suggest that activation of these cells is associated with their extravasation into peripheral lymphoid tissues. Transient lymphopenia following IV N-BP administration has previously been reported [1, 2]; this suggests that the proinflammatory cascade of cytokines triggered by γδ T cells promotes a more general extravasation of lymphocytes from the peripheral circulation.

In patients naive to N-BP the significant decline in total T cells observed within 2 days of N-BP administration disappeared 1 year later. Indeed, baseline total T-cell number was very similar in patients naive or not naive to N-BP (Table 1), and it did not significantly change after ZOL infusion in N-BP-exposed patients (Fig. 1). T-cell populations homeostatically expand to fill available niches, so it is not surprising that this cell count recovered completely. In this study we documented that this decline recovered for most circulating lymphocytes but not for γδ T cells.

The long-term effect of N-BP treatment on circulating γδ T cells has never been reported. The observation that the effect is persistent and may occur also after treatment with oral N-BPs has important clinical implications.

Recently, we observed that the proportion of circulating γδ T cells is an important determinant of the occurrence of the APR after IV infusion of the N-BP ZOL [11]. The findings of the present study link the two observations: the long-lasting desensitization for the occurrence of the APR after repeated IV N-BPs [1, 10] and previous use of oral bisphosphonates [10], confirmed here, are related to the ability of both oral and IV N-BPs to persistently lower circulating γδ T cells.

This long-lasting decrease in circulating $\gamma\delta$ T cells may be attributed to the already mentioned activation, differentiation, and homing at the tissue level of $\gamma\delta$ T cells by the N-BPs [18]; but an effect of inhibition of osteoclast function on the hematopoietic stem cell [19] cannot be ruled out.

The loss of reactivity to N-BPs remains unexplained, and further studies to investigate changes in the reactivity of $\alpha\beta$ and $\gamma\delta$ T cells upon initial and repeat exposures are warranted.

The implication of our observation of a persistent decrease in circulating $\gamma\delta$ T cells is potentially extremely important. The function of $\gamma\delta$ T cells was associated with resistance to tumors [20]. If the long-lasting decrease in circulating $\gamma\delta$ T cells induced by N-BP treatment is an expression of homing at the tissue level, our observations lend support to the reported associations between treatment with N-BPs and the protection against a number of tumors [21–25].

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