Original Articles

Peri- and intratumoral T and B lymphocytic infiltration in breast cancer

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Summary

Purpose: To investigate peritumoral and intratumoral infiltrates in surgical specimens obtained from patients with invasive breast cancer, and of relating these to tumor size. *Methods:* Twenty-six surgical specimens obtained from patients diagnosed with breast cancer underwent immunohistochemical preparation and CD3, CD8, CD20 and CD68 labeling. The positive cells were counted in the tissue samples and correlated with the tumor size determined by imaging methods (TIA ≤ 2 or TIB > 2 cm). *Results:* There was a significant reduction in intratumoral B lymphocytes (CD20+), although this reduction could only be observed in TIA. In relation to peritumoral T lymphocytes (CD3+), there was a significant reduction in TIB, in comparison with TIA. Peritumoral and intratumoral CD3+ and CD68+ presence in completely opposite ways in both sizes of tumors. *Conclusion:* Peritumoral and intratumoral infiltrates of T and B lymphocytes are different and depend on tumor size.

Key words: Infiltration cells; Lymphocytes; Macrophages; Neoplasia; Immunohistochemistry.

Introduction

Since the first research on tumors and the immune system, studies have attempted to correlate cell infiltrates with the immune response to tumors in several organs [1, 2], including in breast cancer cases [2, 3]. It was thus hoped to gain a better understanding of the specific mechanisms through which the immune system may respond to tumors. Through such understanding, it might at some time in the future become possible to interfere with the pathogenesis of tumor cells and induce their eradication. One of the most interesting findings regarding lymphocytic infiltrates in peritumoral tissue and peripheral blood is the increase in the CD4+/CD8+ ratio in patients with breast cancer [1, 2]. Breast cancer patients present suppressed neutrophil migration response upon chemotherapy, and this is probably due to the circulation of factors involved in mechanisms that inhibit neutrophil migration [4]. There has been speculation that these cells may have an antitumor function, but this has not yet been confirmed [5].

Another question that needs further study is whether inflammation and lymphocytic infiltration occur in order to favor the antitumor response to breast cancer, or not. Tumors with markers indicating a worse prognosis have been shown to exhibit increased quantities of lymphocytic infiltrates, and of natural killer (NK) cells in particular [6]. A study on precursors by Underwood concluded that lymphocytic infiltration is a sign of favorable prognosis [7]. Corroborating this hypothesis, Ogmundsdóttir *et al*. showed that lymphocytes may stimulate the epithelium of breast cancer, and that this stimulation is strongly correlated with the expression of class I MHC molecules (major histocompatibility complex) molecules by the tumor cells [8]. In contrast, there are other studies demonstrating that leukocytic inflammation and infiltration are associated with a worse prognosis [9, 10]. Stewart and Tsai [11], in a wide-ranging review of 35 independent studies, found that in 23 of these a high degree of lymphocytic infiltration in tumors was correlated with worse prognosis for the patients.

The role of immune response in patients with breast cancer is still a source of controversy with regard to its relationship with prognostic factors and its relevance, to the point that the response may alter the way in which the therapy is conducted. Another point that should be considered is tumor size versus cellular type (intra- and peritumoral). Independent of their size, different factors and/or different concentrations could be secreted that might induce or inhibit the selective migration of different intra- and peritumoral cell types. Thus, the present study had the aims of studying peritumoral and intratumoral infiltrates of T and B lymphocytes and macrophages in biopsies obtained from patients with invasive breast cancer, and of relating these to tumor size.

Materials and Methods

Patients

Twenty-six surgical specimens from female patients (26 patients in total) with an anatomopathological diagnosis of invasive breast cancer were analyzed. The patients were attended by the mastology team of the Teaching Hospital of the Federal University of Triângulo Mineiro.

Revised manuscript accepted for publication October 29, 2007

Fig. 1a

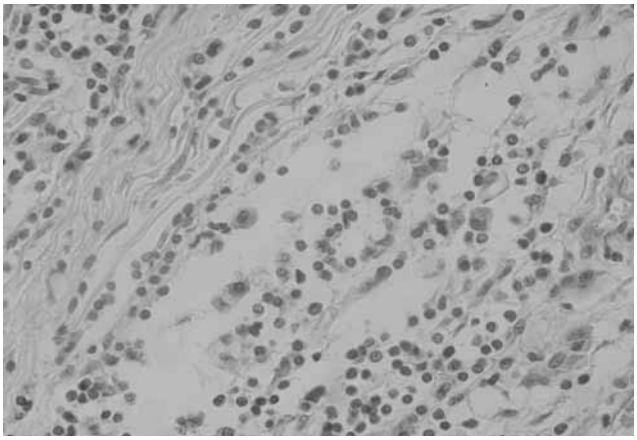

Fig. 1b

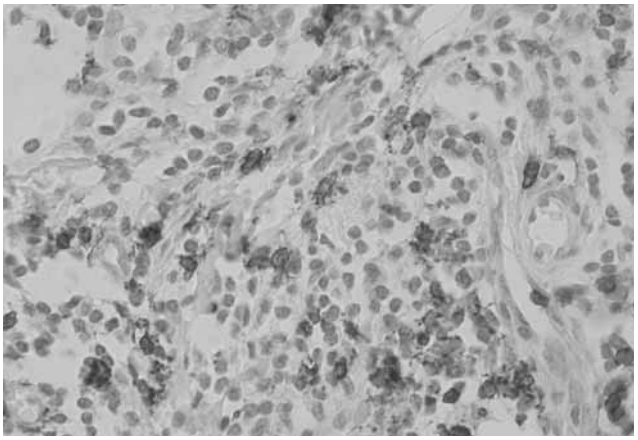

Fig. 1c

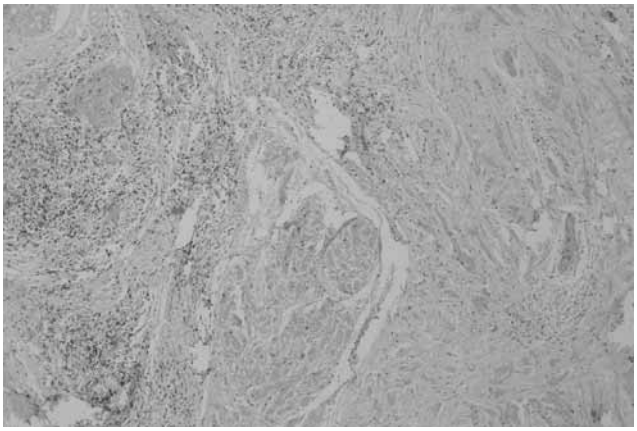

Figure 1a-c — respectively, CD68 (400x), CD20 (400x) and CD8 (40x) with rare, moderate and abundant cells stained as the scoring standard adopted by Georgiannos *et al*. [14].

Patient ages ranged from 26 to 81 years (x = 50.6 ± 11.3), parity from one to ten deliveries (x = 3 ± 2.2), age at the time of menarche from ten to 16 years (x = 13.1 ± 1.5) and age at the time of first pregnancy from 16 to 44 years (x = 24 ± 6.8). All 26 patients had children and 23 had breastfed, with length of breastfeeding ranging from two to 120 months (x = 24.4). Age at the time of menopause (n = 13) ranged from 40 to 55 years (x = 47.2 ± 4.7).

The predominant histological type was invasive ductal carcinoma, which accounted for 21 (80.8%) of the 26 cases studied. There were also two cases of invasive lobular carcinoma, one of tubular-lobular carcinoma, one of inflammatory carcinoma, and one of mixed ductal and adenosquamous carcinoma.

Tumor size

The average diameter of the tumor ranged from 2.0 to 7.0 cm. Most of the tumors were in clinical Stage IIB (n = 18), and there were also three cases in each of Stages I and IIIA, and one case in each of Stages IIA and IIIB.

Patients were assessed at the time of diagnosis using imaging methods (mammography and ultrasound). Longitudinal and transverse diameters were obtained and the greatest diameter was considered for the staging. Clinical staging of the disease was based on the TNM system, as updated by the Union Internationale Contre le Cancer (UICC) [12] and the American Joint Committee on Cancer (AJCC) [13]. Patients with Stage IV were excluded from the study. Mammography and ultrasound (US) were used for tumor measurement, because the clinical measurement method involves normal breast tissue and, if peritumoral edema were present, this could overestimate the size. Histopathologic size was not used because some patients were submitted to neoadjuvant chemotherapy. Image tumor sizes (TI) were divided for statistical purposes in tumors less than or equal to 2 cm (TIA, n = 17) or tumors larger than 2 and < 6 cm (TIB, n = 9).

Anatomopathological analysis

The products from the surgical specimens utilized for diagnosing the neoplasia were fixed in 10% formol. All the specimens contained representative samples of the tumor. Next, the material was subjected to dehydration in increasing concentrations of alcohol, from 70% to pure alcohol (100%), and was then washed with xylol. The specimens were then immersed in liquid paraffin at 60°C and subsequently placed in a rectangular receptacle, in which the material was left to solidify at room temperature, thereby forming a paraffin block. Serial sections of 5 µm in thickness were cut from the paraffin blocks using a microtome and prepared on slides with cover slips for observation under an optical microscope.

Immunohistochemistry and cell counting technique

The slides were deparaffinized using xylol and immediately hydrated in pure alcohol and subsequently in water. They were immersed in phosphate-buffered saline (PBS) solution (pH 6) and left in 3% oxygenated water for 15 min. Next, they were placed in a steaming pan for 30 min with a citrate buffer and then cooled, rinsed, washed and left in PBS solution for 10 min. The slides

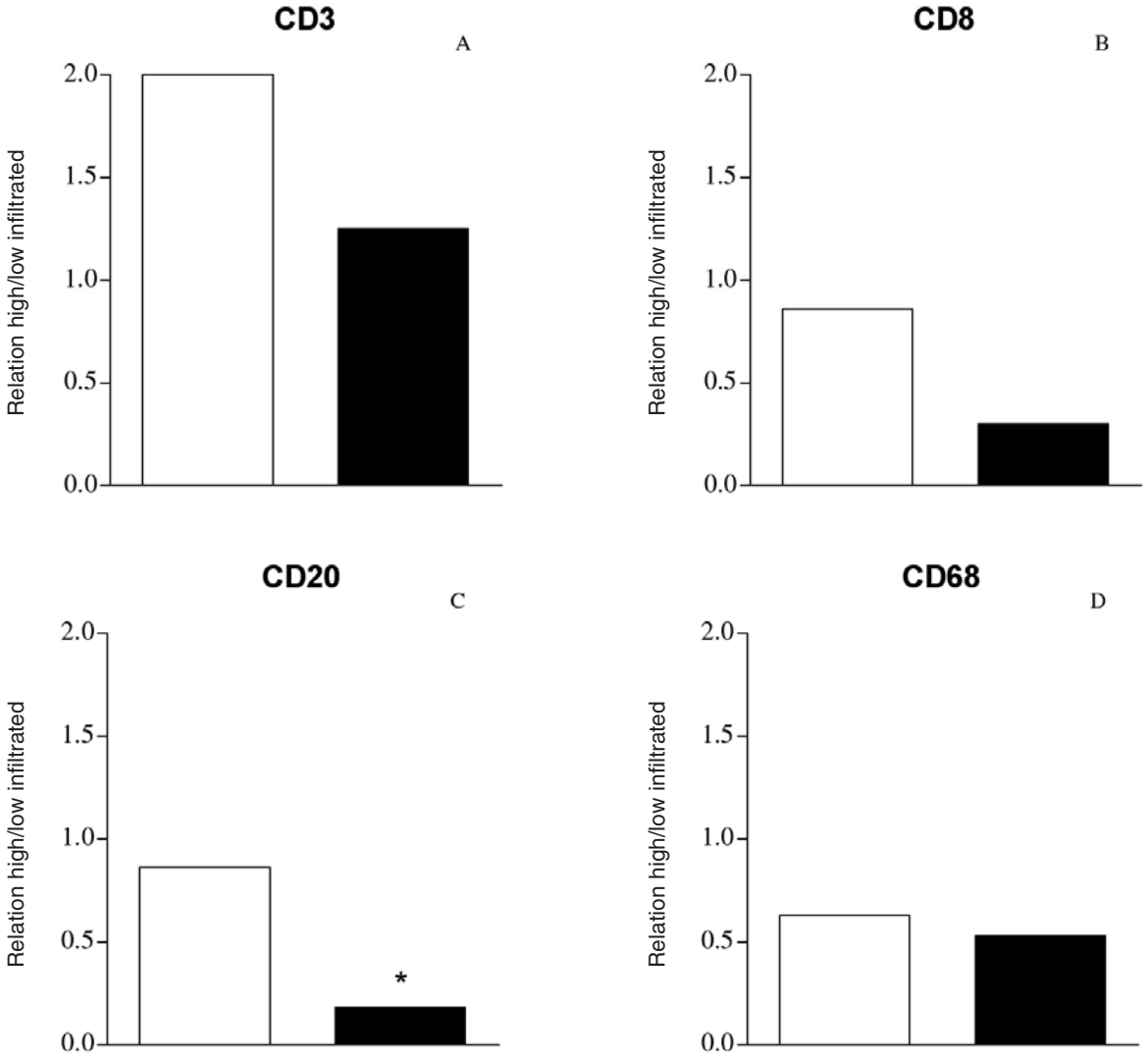

Figure 2. — Distribution of different cell types according to the intensity of peritumoral and intratumoral infiltrates. The intensity of immune cell infiltrates in 26 biopsies obtained from patients with breast cancer was evaluated by immunohistochemistry and determined in accordance with the protocol devised by Georgiannos *et al*. The different cell types studied were: T lymphocytes (CD3 - A) cytotoxic lymphocytes (CD8 – B) B lymphocytes (CD20 – C) and macrophages (CD68 – D). The results were expressed as infiltrate intensity ratios (high/low), according to cell localization: peritumoral (□) or intratumoral (■). * p = 0.03 (Fisher test), comparing peritumoral and intratumoral CD20.

were then incubated for 20 hours with the primary antibodies: CD3 (polyclonal, subclass IgG1/Kappa, dilution 1:800; Dako), CD8 (clone 1A5, subclass IgG1/Kappa, dilution 1:50, Dako), CD20 (clone L26, subclass IgG1/Kappa, dilution 1:600, Dako) and CD68 (clone KP1, subclass IgG1/Kappa, dilution 1:1000; Dako). All these were diluted in 1% PBS-BSA (phosphate-buffered saline solution containing 1% bovine serum albumin). Afterwards, they were then washed twice with PBS and incubated with the secondary antibody (antirabbit or antimouse biotinylated antibodies) for 30 min. They were again washed and then incubated with peroxidase-conjugated streptavidin (Dako LSAB 2 peroxidase kit) for 30 min. Finally, the slides were washed and developed using diaminobenzidine solution for 5 min and mounted for analysis, using Entellan. The same technique was applied to lymphoid tissue (amygdala), as a control.

Following these immunohistochemical procedures, the distribution patterns and morphological details of the cells were analyzed and compared, and carefully recorded for each specimen. The scoring standard adopted by Georgiannos *et al*. [14] was utilized for counting the lymphoid cells. Briefly, the slides were categorized according to the proportion of stained cells: 0 = none; 1 = rare cells; 2 = moderate number of stained cells; 3 = abundance of stained cells (Figure 1a-c). Initially, the cells were observed at low magnification (10x), to obtain a general impression of the cell distribution, and in particular the maximum count. Following this, they were examined in detail (magnification: 40x) to obtain the final counts. These procedures were done by means of analyzing the peritumoral or intratumoral cells that were labeled. Intratumoral study was analyzed in neoplastic tissue without necrosis, and the peritumoral as normal

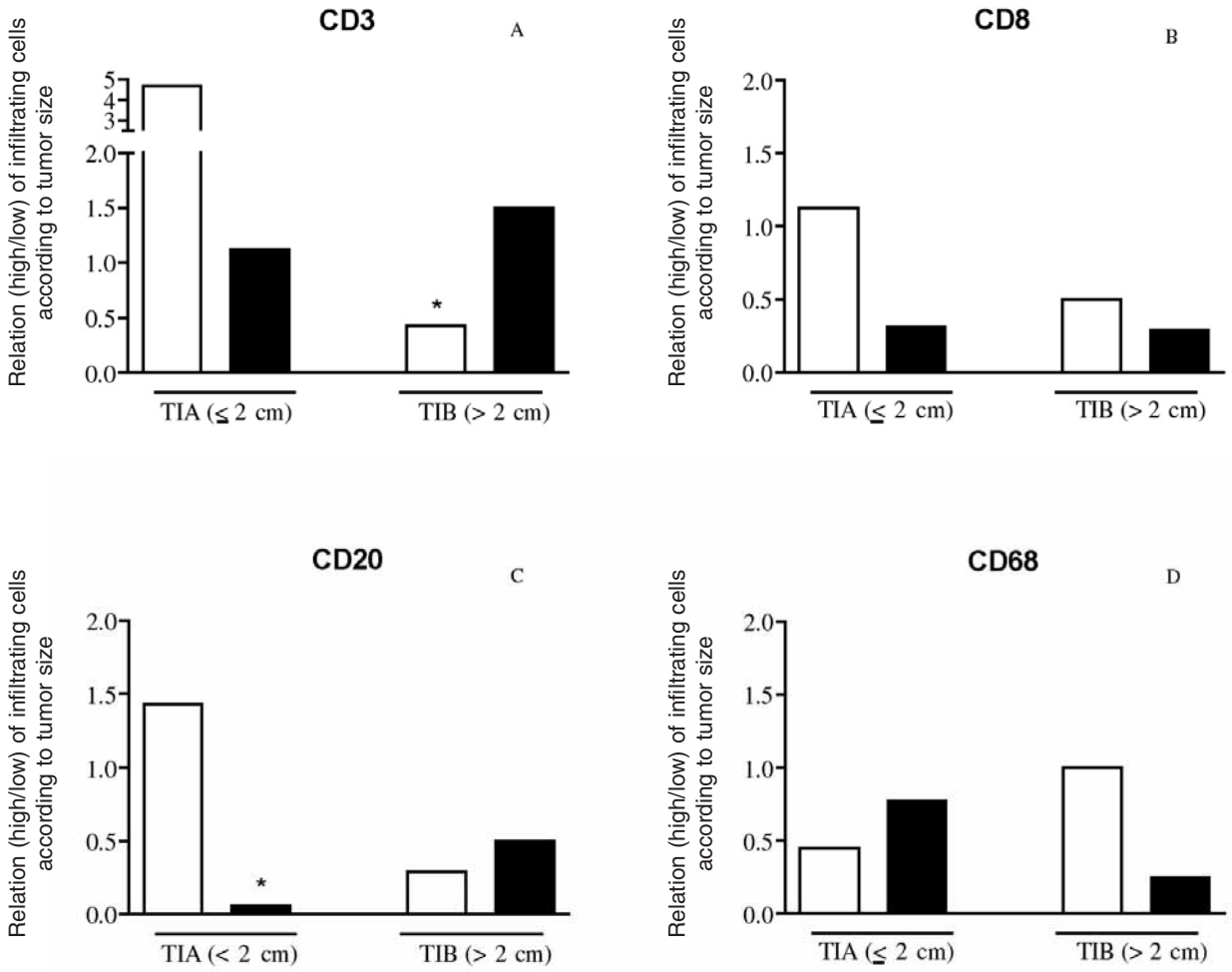

Figure 3. Distribution of different cell types between peritumoral and intratumoral localization, according to tumor size. The intensity of immune cell infiltrates in 26 biopsies obtained from patients with breast cancer was evaluated by immunohistochemistry and determined in accordance with the protocol devised by Georgiannos *et al*. Tumor size was determined by imaging methods (TI) and grouped as less than or equal to 2 cm (TIA, n = 17) or larger than 2 cm (TIB, n=9). The different cell types studied were: T lymphocyte (CD3 – A), cytotoxic lymphocyte (CD8 – B), B lymphocyte (CD20 – C) and macrophage (CD68 – D). The results were expressed as infiltrate intensity ratios (high/low), according to cellular localization: peritumoral (□) or intratumoral (■). • p = 0.0127 (Fisher test), comparing peritumoral CD3 in TIA and TIB tumors. * p = 0.0024 (Fisher test), comparing CD20 in TIA tumors between peritumoral and intratumoral localization.

breast tissue around the neoplasia. Infiltrations of lymphoid cells in regions distant from the neoplastic focus were not taken into consideration.

Statistical Analysis

Analysis of the slides was performed on the final result obtained following concordance between three observers. The slides were categorized for analysis according to the proportion of stained cells: low infiltration when the proportion was 0 and 1 (respectively, none and rare stain cells), and high infiltration when the proportion was 2 or 3 (respectively, moderate and abundance of stain cells). The results are presented as the ratio of the numbers of high cases/low cases (2-3 stain cells/0-1 stain cells). The Fisher test was used for statistical analysis (GraphPad Prism version 3.00-GraphPad Inc., San Diego, CA). Results were considered to be statistically significant when $p \leq 0.05$.

Results

Figure 2 shows the intensities of infiltrated T lymphocytes – CD3 (A), cytotoxic lymphocytes – CD8 (B), B lymphocytes – CD20+ (C) and lymphocytes and macrophages – CD68 (D), according to localization in relation to the tumor (peritumoral or intratumoral). Intratumoral CD3, CD8, CD20 were less stained, and CD68

was similar. There was a statistically significant difference between the peritumoral and intratumoral intensities of B lymphocytes.

Figure 3 presents the intensities of the labeled immune cells according to two parameters: localization of the infiltrate (peritumoral or intratumoral) and tumor size (TIA or TIB). It can be seen that the results shown in Figure 2 are attributed to tumor size. A significant difference was observed for peritumoral T lymphocytes, since TIA presented a reduction in comparison to TIB. Another significant difference was seen in relation to the CD20 marker for tumors sized less than or equal to 2 cm: peritumoral infiltrates presented a greater number of cells than did intratumoral infiltrates. There were lower staining counts of intratumoral CD8 lymphocytes in both TIa and TIB, and lower staining of intratumoral CD68 in TIB.

Discussion

Infiltration by immune cells is a common feature in many human tumors, and it has been suggested that the degree of infiltration is a measure of the host immune response. Several studies have attempted to correlate the infiltrate with the prognosis of breast cancer. Another question is the difference in peri- or intratumoral lymphocyte infiltration. It is an interesting point that immune cells sometimes migrate to the peritumoral site, but the infiltration is different in the intratumoral site. Is it possible that some cytokines or mediators produced by neoplasic cells could inhibit the migration of some types of cells, and thus tumors that have this evasion mechanism have a poor prognosis? Menard *et al*. analyzed 1,919 cases of primary ductal and lobular infiltrating breast carcinomas from women with long-term follow-up and showed that 16-17% of the tumors presented infiltrate that was independent of the patient's age at diagnosis. However, they were unable to find any correlation between the infiltrate and the prognosis [3].

Analysis of the infiltrate in our study showed frequent presence of the four cell types studied. T CD3+ lymphocytes were in most cases present in higher infiltrates, while T CD8+ lymphocytes were found in lower infiltration. These data are in agreement with a previous study [1]. In a study on 60 cases of malignant breast neoplasia, Georgiannos *et al*. [14] did not find any cases of category 0 for CD3+. The CD3+ lymphocytes were present in high infiltration in all TIB cases, while CD8+ in these cases was more frequently found in lower infiltration. T CD8+ lymphocytes were present at lower concentrations when the patients presented tumors > 2 cm. As CD8+ T lymphocytes were also present with CD3+, we could deduce that the cells stained by CD3 antibody and not by CD8 could be CD4+ T lymphocytes.

Campbell *et al*. [15] investigated the profile of intracellular T cell cytokines in the peripheral blood of patients with breast cancer. They found a significant reduction in the percentages of CD4+ and CD8+ cells that were producing IL-2, IFN-gamma, TNF-alpha and IL-4, in comparison with the control group of healthy individuals. Patients with breast cancer have been found to present lower absolute numbers of lymphocytes in the peripheral blood [16]. In contrast, higher numbers of suppressive Treg CD4+ CD25+ lymphocytes are present in the peripheral blood and tumoral microenvironment [17, 18].

In the present study, it was also observed that T CD3+ and CD8+ lymphocytes had an important participation in the tumoral microenvironment. Although there was a large presence of infiltrate containing these lymphocytes, in relation to the presence of others, it was ineffective in eradicating the primary tumor. The presence of T CD3+ lymphocytes in the infiltrate was more frequent in tumors of more than 2 cm in diameter. Nonetheless, some studies still question whether the infiltrate is related to favorable evolution of the disease [9, 10]. However, the present data suggest that the bigger the tumor is, the greater the immune response is, as represented by T CD3+ lymphocytes. This therefore suggests that larger infiltrations by intratumoral T lymphocytes could be related to less favorable prognosis.

B lymphocytes, which in the present study were labeled with CD20+ antibodies, have been frequent findings in peritumoral infiltrates [19-22]. Nevertheless, our data showed that this occurred only in TIA, while in TIB there was increased intratumoral CD20. There was no relationship between the levels of B lymphocytes and clinical stage, which has also been found in other studies [22], or with the other variables studied. B lymphocytes produce antibodies that bond to various antigens. When they are present in intratumoral infiltrates, they produce antitumor antibodies and inhibit the growth of autologous tumor cells, although this response is not limited to specific tumor antigens [23]. However, the majority of these antibodies are against auto-antigens and not just against tumor antigens [20].

Another cell type studied was macrophages. Interestingly, these cells presented an inversion that was dependent on tumor size: TIA had more high intratumoral infiltrate than peritumoral infiltrate, while TIB had more peritumoral macrophages than intratumoral macrophages. The presence of macrophages was the opposite of what was observed for T lymphocytes (CD3+). These data are similar to what is presented in the literature. Ben-Hur *et al*. [24] studied 17 cases of invasive breast carcinoma and, in evaluating the CD68+ macrophages, found few cells in different areas of the tumor. There is uncertainty regarding the function of the presence of macrophage infiltrates, but an association between poor prognosis and severe macrophage infiltrate in breast cancer cases has been found in several studies [24-26].

Studies on animal models have demonstrated that infiltrating macrophages in the tumor induce apoptosis in T CD8+ cells by means of a mechanism that requires cell contact and mediation by tumor necrosis factor (TNF) and nitric oxide [27]. High levels of macrophages in focal areas of the tumor are associated with increased vascular density, and the groups of macrophages are found in avascular areas of the tumor, which is associated with worse prognosis [28]. Aggressive tumors rapidly increase

their vascular supply in some areas, while leaving other areas in prolonged hypoxia that subsequently leads to necrosis. This might attract macrophages to the interior of the tumor, to contribute to the process of angiogenesis [29]. Disease-free survival and overall survival are worse when there are large quantities of macrophages [29-31]. As the evolution of long-term cases becomes available, we may be able to verify the prognosis for such patients.

Conclusion

Taken together, peri- and intratumoral infiltration may contribute to tumor regression or progression. Another interesting finding that deserves further study is the infiltration of T lymphocytes and macrophages. However, these findings are very complex and also require further studies. In the present study our aim was to demonstrate that tumor size might be related to peritumoral and intratumoral immune cell infiltrates in breast cancer cases. In conclusion, our results suggest that peritumoral and intratumoral infiltrates of T and B lymphocytes are different and depend on tumor size.

Acknowledgements

We thank FAPEMIG, FINEP and CNPq for funding.

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