Long-term monitoring of cell-mediated immunity in disease-free breast cancer patients: a preliminary retrospective study

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Abstract

In 102 N– and 44 N+ disease-free breast cancer patients, lymphocytic populations and skin reaction of delayed hypersensitivity (SRDH) were monitored up to 266 months after mastectomy to find out whether they were similar or different from control values. In two selected groups of 34 N– and 11 N+ breast cancer patients, the whole 10 year follow-up was divided into three subintervals, each of them lasting 40 months and the time course of lymphocytic populations was evaluated. In the 102 N– patients, mean CD4+, CD8+, CD3+ values were lower ($P < 0.01$, $P < 0.001$, $P < 0.01$, respectively) while CD4+/CD8+ ratio was higher ($P < 0.05$) than in controls. Fifteen N– breast cancer patients (16%) were anergic compared to 30 (32%) of controls ($P < 0.05$). In the 34 selected N– breast cancer patients soon after mastectomy the mean value of CD4+, CD8+, CD3+ T subpopulations was lower ($P < 0.01$, $P < 0.001$, $P < 0.01$, respectively) than in controls. Successively their mean value increased so that in the last subinterval they were not or were only slightly lower ($P$ n.s., $P < 0.05$, $P < 0.05$, respectively) than in controls. In the 44 N+ patients, mean CD4+, CD8+, CD3+ values were lower ($P < 0.001$, $v < 0.05$, $P < 0.01$, respectively) and CD19+ lymphocytes higher ($P < 0.001$) than in controls. Five N+ breast cancer patients (13%) were anergic compared to 32% of controls ($P < 0.05$). In the 11 selected N+ breast cancer patients soon after mastectomy, the mean value of CD4+, CD8+ T subpopulations and CD16+56+ cells was significantly lower ($P < 0.001$, $P < 0.001$, $P < 0.01$, respectively) than in controls. Successively their mean value constantly increased so that in the last subinterval, no or slight ($P$ n.s., $P < 0.05$, $P$ n.s., respectively) significant difference compared to controls occurred. The mean CD4+/CD8+ ratio value of N– patients was significantly higher than in controls. However in the last subinterval, the significance was lower than in the first one ($P < 0.05$ and $P < 0.01$, respectively). In the N+ patients, the mean value of CD4+/CD8+ ratio was constant, although not significantly, lower than in controls; however it progressively increased from the first to the last subinterval. Therefore the significance of the difference of the mean CD4+/CD8+ ratio between N– and N+ patients strongly decreased from the first to the last subinterval ($P < 0.001$ and $P < 0.05$, respectively). These data indicate that in breast cancer patients, following mastectomy, a significant activation of memory and CD4+ T cells and long-term decrease of the circulating immunocompetent CD4+, CD8+ and CD16+56+ cells occurs. The prolonged disease-free interval observed in the 34 N– and 11 N+ breast cancer patients can be correlated with the restoration of the normal state of cell-mediated immunity.

Keywords: Breast cancer; Disease-free; Cell-mediated immunity

1. Introduction

In breast cancer patients, immunosuppression after removal of primary tumour and low plasma circulating CD4+/CD8+ ratio values at the relapse have been reported [1-6]. In some subsets treated with immunosuppressive drugs, dose-dependent decrease of metastatic lesions [7] and prolonged survival in patients submitted to post-operative complementary radiotherapy [7,8], which is immunosuppressive, have been observed. More recently it was demonstrated that the cytokines from mononuclear cells infiltrating the neoplastic tissue can stimulate or suppress tumour growth [7,9-11].
These fragmentary and contradictory findings do not permit any definition of the role of cell-mediated immunity (CMI) in breast cancer.

In this study, lymphocytic populations and skin reaction of delayed hypersensitivity (SRDH) were monitored in breast cancer patients disease-free for a prolonged time after mastectomy. The purpose of the study was to investigate whether in non-relapsed breast cancer patients CMI and SRDH were similar to or different from control values and whether CMI showed a different time course in the two patient groups with different prognosis (N+ and N−).

2. Materials and methods

2.1. Patients and follow-up

From January 1989 to December 1999, 146 breast cancer patients who were regularly followed up for a prolonged time after mastectomy also were submitted to concomitant immunological study. At the post-operative histology, 44 patients showed axillary lymph-node involvement (N+) and the 102 remaining did not (N−). In the 146 breast cancer patients, the duration of the clinical follow-up from mastectomy and of the immunological study was 148 ± 55 and 85 ± 34 months (mean ± S.D.), respectively. During the immunological study T subsets, CD16+56+ cells, B lymphocytes and SRDH were determined in 93 (91%) of the 102 N− and in 39 (89%) of the 44 N+ breast cancer patients. In the remaining 9 N− and 5 N+ patients, only T subsets, CD16+56+ cells and B lymphocytes were evaluated. Besides in these 146 breast cancer patients, all immunological determinations carried out when the patient was under adjuvant tamoxifen or up to 3 years following adjuvant chemotherapy were excluded. Therefore the determinations during mastectomy were considered, and in the case of skin reaction at the control site, the test was invalidated. The score consisted of the total obtained by adding the results from all recall antigens. Anergy was defined when the score of all determinations was zero or when in one or more determinations the score was zero and in the remaining the measurable response of one or more antigens did not exceed 2 mm. In controls, in one or more antigens did not exceed 2 mm. In controls, in

2.2. Methods

Heparinized venous blood samples were taken in fasting patients and immediately transferred to the laboratory for measurement. Phenotypic analysis of the main lymphocytic populations such as CD3+ (total T cells), CD8+ (T suppressor/cytotoxic cells), CD4+ (T helper/inducer cells), CD19+ (B lymphocytes), CD56+CD16+ (CD16+56+ cells) was determined by flow cytometry (FACSStar, Becton-Dickinson, Mountain View, CA) using the following monoclonal antibodies combined with phycoerythrin (PE) or fluorescein isothiocyanate (FITC): anti-CD3 (FITC), anti-CD8 (PE), anti-CD4 (FITC), anti-CD19 (PE), anti-CD56 (PE) and anti-CD16 (PE). The parameters were expressed as cells/µl. In addition, CD4+/CD8+ T cell ratio (T4/T8 ratio) was determined. All monoclonal antibodies were provided by Becton-Dickinson. SRDH was evaluated by IMC Multitest (Mérieux) and three parameters were taken into account: number of positive antigens, anergic patients, score. IMC Multitest was performed on the forearm. It included seven recall antigens (tetanus, diphtheria, streptococcus, tuberculin, proteus, tricopyton, candida) and glyc-}

2.3. Statistical analysis

All the CMI determinations that could have been affected by factors other than breast cancer and those under adjuvant therapy were excluded. Therefore the determinations during adjuvant tamoxifen and up to 3 years following adjuvant
Table 1
Mean values (mean ± S.D.) in peripheral blood of T subsets, CD4+/CD8+ ratio, CD16+56+ cells and CD19+ lymphocytes in 146 disease-free breast cancer patients and in 24 controls.

<table>
<thead>
<tr>
<th>PTNM</th>
<th>Patients</th>
<th>Cell-mediated immunity (CMI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(n)</td>
<td>CD4+ (cells/µl)</td>
</tr>
<tr>
<td>N−</td>
<td>102</td>
<td>879 ± 342</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(P &lt; 0.01)</td>
</tr>
<tr>
<td>N+</td>
<td>44</td>
<td>839 ± 343</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(P &lt; 0.001)</td>
</tr>
<tr>
<td>Controls</td>
<td>24</td>
<td>1051 ± 269</td>
</tr>
</tbody>
</table>

* P values: versus controls.

chemotherapy were excluded. The lymphocytic populations and SRDH of the 146 (102 N− and 44 N+) non-relapsed breast cancer patients were computed as the mean value (mean ± S.D.) of all the measurements performed in each patient group. These mean values were compared with those of controls by the unpaired t-test. The percentages of anergic patients in N+ and N− groups were compared with those of controls by the χ² test. In two groups of 34 N− and 11 N+, non-relapsed breast cancer patients who regularly had carried out most scheduled post-operative control visits, the relationship between the values of each immunological parameter of CMI and the corresponding time up to 10 years after mastectomy was explored. In fact in these two N− and N+ breast cancer groups, the whole 10 year follow-up was divided into three subintervals, each of them lasting 40 months. In any but the first of the three subintervals, a similar number of determinations of the immunological parameters were considered. In the first subinterval, all determinations carried out under tamoxifen or within 3 years after adjuvant chemotherapy were ruled out from statistical analysis. Therefore relatively few determinations were taken into account and the mean value (mean ± S.D.) of CD3+ T subpopulations was computed only in the last two subintervals. In each interval, we calculated the mean value (mean ± S.D.) of each parameter that was ordered with time from mastectomy; thereafter the mean of these individual values in the first subinterval was compared by unpaired t-test with the corresponding ones obtained in the last subinterval and they both were separately compared with that of control subjects. Moreover the mean values of the parameters obtained in the first and in the last subintervals in the 34 N− patients were compared with the corresponding ones obtained in the same subintervals in the 11 N+ patients.

3. Results

3.1. CMI, B lymphocytes and SRDH (Tables 1 and 2)

Table 1 compares the mean ± S.D. values of all the determinations of T subsets, CD4+/CD8+ ratio, CD16+56+ cells, B lymphocytes in 102 N− and 44 N+ disease-free breast cancer patients with those of the 24 controls. In the 102 N− patients, mean CD4+, CD8+, CD3+ values were significantly lower (P < 0.01, P < 0.001, P < 0.01, respectively) and mean values of CD4+/CD8+ ratio and B lymphocytes were significantly higher (P < 0.05) than in controls. No significant difference occurred with regard to CD16+56+ cells. Also in the 44 N+ patients, mean CD4+, CD8+, CD3+ values were significantly lower (P < 0.001, P < 0.05, P < 0.01, respectively) and B lymphocytes significantly higher (P < 0.001) than in controls. In these patients, no significant difference occurred with regard to CD4+/CD8+ ratio and CD16+56+ cells.

Table 2 compares the score and the number of positive antigens of the 93 N− and 39 N+ non-relapsed breast cancer patients with controls. In the 93 N− patients, the number of
Table 3
Mean values (mean ± S.D.) in peripheral blood of T subsets, CD4+/CD8+ ratio, CD16+56+ cells and CD19+ lymphocytes in three subintervals during the post-operative follow-up of 34 disease-free N- breast cancer patients

<table>
<thead>
<tr>
<th>Time after mastectomy (months)</th>
<th>CD4+ (cells/µl)</th>
<th>CD8+ (cells/µl)</th>
<th>CD3+ (cells/µl)</th>
<th>CD4+/CD8+ ratio</th>
<th>CD16+56+ (cells/µl)</th>
<th>CD19+ (cells/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up to 40</td>
<td>834 ± 307</td>
<td>418 ± 155</td>
<td>1240 ± 443</td>
<td>2.17 ± 0.94</td>
<td>164 ± 157</td>
<td>169 ± 97</td>
</tr>
<tr>
<td>41–80</td>
<td>946 ± 355</td>
<td>460 ± 200</td>
<td>1457 ± 501</td>
<td>2.25 ± 0.99</td>
<td>129 ± 105</td>
<td>195 ± 107</td>
</tr>
<tr>
<td>81–120</td>
<td>1000 ± 352</td>
<td>513 ± 208</td>
<td>1442 ± 473</td>
<td>2.1 ± 0.83</td>
<td>214 ± 104</td>
<td>190 ± 121</td>
</tr>
<tr>
<td>Controls</td>
<td>1051 ± 269</td>
<td>643 ± 203</td>
<td>1617 ± 408</td>
<td>1.79 ± 0.7</td>
<td>188 ± 82</td>
<td>143 ± 67</td>
</tr>
</tbody>
</table>

Unpaired t-test: * versus controls; ** versus up to 40 months.

Table 4
Mean values (mean ± S.D.) in peripheral blood of T subsets, CD4+/CD8+ ratio, CD16+56+ cells and CD19+ lymphocytes in three subintervals during the post-operative follow-up of 11 disease-free N+ breast cancer patients

<table>
<thead>
<tr>
<th>Time after mastectomy (months)</th>
<th>CD4+ (cells/µl)</th>
<th>CD8+ (cells/µl)</th>
<th>CD3+ (cells/µl)</th>
<th>CD4+/CD8+ ratio</th>
<th>CD16+56+ (cells/µl)</th>
<th>CD19+ (cells/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Up to 40</td>
<td>547 ± 190</td>
<td>344 ± 169</td>
<td>–</td>
<td>1.69 ± 0.26</td>
<td>80 ± 79</td>
<td>143 ± 109</td>
</tr>
<tr>
<td>41–80</td>
<td>790 ± 295</td>
<td>451 ± 131</td>
<td>1091 ± 320</td>
<td>1.81 ± 0.64</td>
<td>144 ± 95</td>
<td>197 ± 95</td>
</tr>
<tr>
<td>81–120</td>
<td>916 ± 428</td>
<td>524 ± 223</td>
<td>1496 ± 611</td>
<td>1.87 ± 0.75</td>
<td>200 ± 176</td>
<td>226 ± 144</td>
</tr>
<tr>
<td>Controls</td>
<td>1051 ± 269</td>
<td>643 ± 203</td>
<td>1617 ± 408</td>
<td>1.79 ± 0.7</td>
<td>188 ± 82</td>
<td>143 ± 67</td>
</tr>
</tbody>
</table>

Unpaired t-test: * versus controls; ** versus up to 40 months; *** versus up to 40 months and **** versus 81–120 months in the 34 N- patients.

positive antigens was not significantly lower from controls, unlike the mean score that was (P < 0.01). Fifteen breast cancer patients (16%) were anergic compared to 30 (32%) of controls (P < 0.05). In the 39 N+ patients, the number of positive antigens was similar while the mean score was significantly lower (P < 0.05) than in controls. Five breast cancer patients (13%) were anergic compared to 32% of controls (P < 0.05).

3.2. CD4+/CD8+ ratio, T subsets, CD16+56+ cells and B lymphocytes: time course in the post-operative follow-up (Tables 3 and 4)

Table 3 shows that in 34 N- breast cancer patients regularly followed up to 10 years after mastectomy, in the first subinterval, the mean value of CD4+, CD8+, CD3+ T subpopulations and CD4+/CD8+ ratio was significantly lower (ranging from P < 0.01 to P < 0.001) unlike CD19+ population and CD16+56+ cells that were not significantly different from the control group. In the third subinterval, CD8+ and CD3+ T subpopulations were significantly lower (P < 0.05) while CD4+/CD8+ ratio and CD19+ population were significantly higher than in the control group (P < 0.05 and P < 0.001, respectively). No significant difference occurred between the mean values of the CD4+ T subpopulation and CD16+56+ cells observed in this last subinterval and those of controls. From the first to the last subinterval, a constant progressive increase of the mean values of CD4+ and CD8+ T subpopulations occurred. The mean value of both CD4+ and CD8+ T subpopulations was significantly higher in the third final subinterval than in the first one (P < 0.001). Also mean value of CD3+ subpopulation and CD16+56+ cells was significantly higher (P < 0.05 and P < 0.01, respectively) in the third compared to the first subinterval. No significant difference of CD4+/CD8+ ratio and CD19+ population occurred in the three subintervals.

Table 4 shows that in 11 N+ breast cancer patients regularly followed up to 10 years after mastectomy, in the first subinterval (or the second for CD3+), the mean value of CD4+, CD8+, CD3+ T subpopulations and CD16+56+ cells was significantly lower (P < 0.001, P < 0.01, P < 0.001 and P < 0.01, respectively) than in controls; the CD19+ population and CD4+/CD8+ ratio were not significantly different from the control group. In the third subinterval, the CD8+ T subpopulation was significantly lower (P < 0.05) and the CD19+ population was significantly higher (P < 0.001) than in control group. No significant
difference occurred between the mean values of CD4+, CD3+ T subpopulations, CD4+/CD8+ ratio and CD16+56+ cells observed in the last subinterval compared to those of controls. From the first (or the second for CD3+) to the last subinterval, a constant progressive increase of CD4+, CD3+, CD19+ populations, CD16+56+ cells and CD4+/CD8+ ratio occurred. The mean values of these populations in the first (or the second for CD3+) were higher than in the third subinterval ($P < 0.001, P < 0.001, P < 0.05$ and $P < 0.01$, respectively); the CD4+/CD8+ ratio was not significantly different. Also mean values of CD8+ T subpopulation were significantly ($P < 0.01$) higher in the last compared to the first subinterval.

In the first subinterval in the 34 N– patients, the mean values of CD4+/T subpopulations, CD16+56+ cells and CD4+/CD8+ ratio were significantly higher than the corresponding mean values observed in the 11 N+ patients ($P < 0.01, P < 0.05, P < 0.001$, respectively). No significant difference occurred with regard to CD8+ and CD19+ populations. In the last subinterval in the 34 N– patients, only mean values of CD19+ population and CD4+/CD8+ ratio were significantly higher ($P < 0.05$) than the corresponding mean values observed in the 11 N+ patients. No significant difference occurred with regard to all the remaining parameters.

4. Discussion

In the 102 N– and in the 44 N+ non-relapsed breast cancer patients, the mean circulating levels of CD4+, CD8+ and CD3+ T lymphocytes were significantly lower than in control subjects (ranging from $P < 0.05$ to $P < 0.001$) (Table 1). In both groups, the values were lower in the first subinterval and tended to increase with time (Tables 3 and 4). Although not significant, also, the mean circulating levels of CD16+56+ cells were lower than in controls (Table 1) and then tended to increase with time (Tables 3 and 4).

A decrease in the total number of T lymphocytes and the immunocompetent cells after major surgery for cancer or benign disease has been reported [16-23] and surgical distress was considered as the reason for this decrease [16-18,24]. However, in these studies, the decrease of T lymphocytes and the immunocompetent cells has been reported only for a few days after surgical distress and the post-operative immunocompetent cell value was not always significantly different from the pre-surgical one [17-19]. Immunosuppression can also occur under adjuvant tamoxifen or chemotherapy and adjuvant CMF has been reported to prolong immunosuppression for one up to three years after it has been discontinued [25-26]. In this study, all determinations of subpopulations and SRDH carried out when patients were under adjuvant therapy and up to three years after chemotherapy were excluded. In spite of that in our N– and N+ breast cancer patients, a marked decrease in the total number of T lymphocytes and the immunocompetent cells compared to normal values occurred.

We already hypothesized that [1] locoregional and/or distant micrometastases can explain the significant decrease of the immunocompetent cells observed following mastectomy. As minimal residual disease likely occurs in most breast cancer patients, this remnant could remain in a dormant state for a long time and in some instances for the whole lifetime. Soon after the excision of the primary tumour, peptide epitopes placed as tumour antigens on the surface of the cellular membrane of the micrometastases may stimulate a specific and not specific cellular immune response mobilizing the immunocompetent CD4+, CD8+ and CD16+56+ cells from blood. In these sites, the immunocompetent cells could play an antitumoural role. A similar hypothesis has been formulated for colorectal cancer metastases [17,27] and it is consistent with the phenotypic analysis of tumour infiltrating lymphocytes (TILs) performed in the metastatic breast cancer tissue. In fact this analysis showed that most TILs are immunocompetent CD4+, CD8+ and CD16+ cells [28-29].

As to CD19+ lymphocytes, a significant increase in N– and in N+ ($P < 0.05$ and $P < 0.001$, respectively) breast cancer patients occurred (Table 1). In both groups, the increase in the last subinterval was significantly different from controls ($P < 0.001$) and it was not in the first one. These findings suggest that in these breast cancer patients, a delayed humoral immunity may have an additive role to CMI and that it is mainly as regards N+ subgroup.

SRDH is mainly due to memory and CD4+ T cells [30]; the significant decrease ($P < 0.05$) of anergic patients in N+ and N– subgroups compared to controls in spite of the significant decrease of the score that was observed in N– and N+ patients can be interpreted by a wide tissue activation of these immunocompetent cells in some of these breast cancer patients.

The different behaviour of the CD4+/CD8+ ratio in the peripheral blood of N– and N+ patients suggests that the decrease of the T cell population tends to be relatively more pronounced for the CD8+ in N– patients and for the CD4+ population in N+ ones. Therefore it can be inferred that after mastectomy in the N– group, more CD8+ than CD4+ cells move from blood to tissues. In the N+ subgroup, the opposite is likely to occur. CD4+/CD8+ ratio had a different time course in N– and N+ patients during the post-operative follow-up. In fact in the N– subjects after mastectomy, the mean value of CD4+/CD8+ ratio was significantly higher than in controls but the significance of difference decreased from the first to the last subinterval ($P < 0.01$ and $P < 0.05$, respectively). In
the N+ patients, the CD4+/CD8+ ratio was constantly, although not significantly, lower than in controls and it progressively increased. In the first and the last subintervals, the mean CD4+/CD8+ ratio value of N– patients was significantly higher than in N+ subjects. However the signification of the difference strongly decreased from the first to the last subinterval (P < 0.001 and P < 0.05, respectively). This tendency of CD4+/CD8+ ratio in N– and N+ breast cancer patients after mastectomy to attain the mean value observed in controls joins with a more favourable prognosis of the studied patients who all were disease-free after a 10 year follow-up.

This study has the following limits: it is a retrospective study, cytokines were not considered, the values of the studied parameters were not determined preoperatively and at fixed times. In spite of that, these data point out that in breast cancer patients, following mastectomy, a significant long-term decrease of the circulating immunocompetent CD4+, CD8+ and CD16+56+ cells, compared to control values, occurs. Moreover significant changes of the immune system occur as indicated by variation of the circulating CD4+/CD8+ ratio and CD16+56+ cells. The prolonged disease-free interval observed in the 34 N– and 11 N+ breast cancer patients can be correlated with the restoration of the normal state of CMI.

References


