Adaptive Immunity in Breast Cancer Iraqian Women

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Abstract: *This study aimed to compare some of the immunological parameters in patients with cancer with the healthy group such parameters include study the level of immunoglobulines IgG, IgM, IgA in different tumor stages and to study the number of CD4 and CD8 and the CD4/CD8 ratio. This prospective study was based on 50 female patients with carcinoma of the breast. The 50 female patients were attending the radiotherapy and nuclear medicine hospital and central public health laboratories. Their ages were ranged from 30 to 72 years, compared with 20 healthy women as a control group. This study was extended through a period from September 2015 to January 2017. The 50 female patients were classified into four groups according to Tumor Node Metastasis (TNM) classification, stage I includes 6 patients, stage II includes 9 patients, stage III includes 16 patients and stage IV includes 19 patients. Highest incidence of breast cancer was at age 40-49, with a mean age of 46 years. The mean serum level of IgA showed no significant difference between patient groups and healthy control group. However mean serum IgG & IgM levels showed a significant increase in patient at stage II, compared to those of the other stages and control group (P=0.014 and P=0.02 respectively). In contrast, patients at stage IV showed decrease of IgM & IgG levels as compared to healthy control group (P=0.093 and P=0.070 respectively). By using direct immunoflourescent assay (IFA), the CD4+, CD8+, T-cells were counted. It was found from the present study that the number of CD4+ cells decrease with the progress of the disease to reach its lowest number at stage IV (P < 0.001), on the other hand, the present results demonstrated opposite finding of CD8 cells that started low in number at early stages of the disease to reach it's highest level at stage IV (P=0.049).*

Keywords: Breast cancer, Immunoglobulines, CD4, CD8

1. Introduction

Breast Cancer: The benign proliferative breast disease is an extremely complex and interrelated group of proliferative disorder of the breast parenchyma, most of which are probably not true neoplasm but rather hormonally induced hyperplastic processes like typical fibroadenoma, other raise the differential diagnosis of carcinoma at the clinical, gross, or microscopic level, and some of them are probably related to the development of malignancy but in a fashion that remains ill-defined and highly controversial. The two key pathological determination to make in the study of breast carcinoma are first whether the tumor is confined to the glandular component of the organ (in situ carcinoma) or it has the invaded the stroma (invasive carcinoma) and second whether it is of ductal or lobular type. Both cellular and humoral mechanisms exhibit anti-tumor activity and have been shown to destroy tumor.[1]

A humoral immune response to polymorphic epithelial mucin (MUC-1) protects against disease progression, and further supports the idea of using synthetic peptides of glycopeptides containing the immunogenic core of the mucin as cancer vaccines [2]. Cell mediated immune response to breast tumor has only been marginally investigated.[3] Clinical studies have shown that a marked lymphoplasmocytic reaction in breast tumors is associated with poor prognosis. Such findings raise the possibility that an inflammatory cell reaction might be a tumor-induced response that tends to promote tumor growth.[4]

2. Literature Review

Breast Carcinoma in Iraqi Patients:
Breast cancer is the most frequent malignancy among women worldwideaccounting for 25% of cancers.[5] According to cancer registry section (Iraqi cancer board) Baghdad / MOH, breast carcinoma is the most common malignant tumor in Iraqi women and it comprise (31.3%) of all female malignant cases. The incidence rate is 16.5 from each 100.000 women in Iraq. The mean age of Iraqi women with breast cancer is 49 year.[6,7,8]

Tumor Immunology:
There is considerable clinical evidence suggesting that tumors are immunogenic and that the human body responds immunologically to tumors in a manner similar to the response to transplanted foreign tissues.
1) The presence of a mononuclear cell infiltrate in situ in inflammatory carcinomas correlates with improved survival rates, for example a person with a breast carcinoma accompanied by an inflammatory response seems to do better than someone whose carcinoma is not associated with an inflammatory process.
2) Metastatic cells commonly are present in patients with cancer, but the frequency of their implantation and growth is low. Two types of immunity, innate and adaptive each of which consist of humoral and cell mediated immunity.[9]

Adaptive Immune Response:
The humoral immunity is mediated by specific antibodies that recognize and react to a challenge; while cell mediated immunity of specific immune response mediated by

lymphocyte (T lymphocyte) which initiate a cellular response through chemical mediators (lymphokines). If an invading antigens escapes the innate defenses, the body can launch an adaptive, or specific response against one type of antigen. Antibodies bind that specific antigen and immobilize it, antibodies are specific for only one antigen. Specialized white blood cells called B cells produce antibodies. B cells must interact with Helper T cells, and other specialized white blood cells, to intiate antibody production.

Cytotoxic T cells kill infected cells, preventing them from producing more antigens. Cytotoxic T cells must interact with helper T cells to regulate destruction of infected cells. Helper T cells regulate other cells of the immune system through secretion of cytokines. [10]

CD4 and CD8 Markers:

The CD4 and CD8 glycoproteins characterized the two main subpopulations of T-lymphocytes. The TCD4 cells are involved in the regulation function (helper/inducer) of the immune response and the TCD8 cells have suppressive and cytotoxic activity. T-function releasing involves the respective recognition of CD4 and CD8 by class II and class I HLA structures which represent their natural ligands.

The count of TCD4 and TCD8 lymphocytes in the peripheral blood is a major test in the hematological follow up of the diseases with immune dysfunction and patients after organ transplant or marrow graft.[11]

The reference technique used for counting TCD4 and TCD8 lymphocytes in the peripheral blood requires the combined utilization of a blood cell counter (to determine the number of total lymphocytes), and flow cytometry. The cells number for each type (TCD4 or TCD8) /microliter of blood are calculated from the product of these two measurements. So, in human, it is determined that the number of T-lymphocyte decrease from birth and stabilize in healthy adult at mean values of 830 * 288 T CD4/ul of blood and 530 * 231 TCD8/ul of blood. [12]

Collection of tumor cells are extensively infiltrated by macrophage and CD8. positive (suppressor / cytotoxic) T cells, but by few CD4. positive (helper) T cells i.e; an altered ratios of CD4 and CD8. [13]

CD3+ T-lymphocytes represented the main population of malignant effusion. Associated with mononuclear cells obtained from patients with metastatic breast cancer, the mean CD4/CD8 ratio was 1.18. [14] Thus tumor infiltrating lymphocytes showed an enrichment of CD8+ cells with a corresponding decrease in CD4+ cells in comparison with peripheral blood lymphocytes. [15]

By studying T-lymphocyte subsets of peripheral blood monocytes, cellular immunity was found suppressed in breast cancer patients with an abnormally elevated percentage of CD8+ T-cell. There was a reversed ratio of CD4/CD8, low T cell proliferation, and some immunity suppressing factors in the peripheral blood. These abnormalities were most evident at stage III of breast cancer patients. The ratio of CD4/CD8 increased significantly and the colony formation of T cells greatly improved 5-6 months after radical mastectomy, it is suggested that immune suppression of tumor is strengthened after surgery.[16] Sequential lymphocyte analysis showed that helper (CD4) and suppressor (CD8) T-cell subsets increased after the first week of treatment and declined thereafter. [17] Other found no relation between clinical response and changes in the CD4/CD8 ratio.[18]

Immunoglobulin:

Antibodies are bifunctional molecules which bind antigen via their V domains and can interact with effector system via their C domains. Antigen binding occurs by the formation of multiple non-covalent bonds between residues in the hypervariable regions of antibodies and those in the antigen.[19] There are two major mechanisms by which antibodies may mediate tumor lysis, the first is through complement fixing antibodies which bind to the tumor cell membrane activating complement then lysing tumor cells.[20] The other mechanism is through antibody defendant cell-mediated cytoxicity in which antibodies usually of the IgG class form an intracellular bridge by binding to a specific determinant on the target cell and the FC receptor of the effector cells.[21]

Singh et al., (1991) reported that serum IgG levels were lower than the normal in patients with breast cancer, after treatment, IgG levels was further decreased. The serum IgA was found to be significantly increased as compared to control. However, after treatment, the IgA level returned to the normal range. Serum IgM level was unaltered in these patients. When compared with the clinical stages, a progressive decrease in serum IgG and increase in IgA was observed with the advancing stage of the disease. Therefore, their observation implies that the disturbance in cell mediated and humoral immunity is associated with the malignant process in the tumor bearing host. [22]

On the other hands, other studies stated that the average concentration of IgG, IgA and IgM fall in the range of normal values during the therapy. Nevertheless, some mild stimulation of IgG production and transient one for IgA can be noticed.[23]

3. Materials and Methods

Patients Group:

A total of 50 female patients with carcinoma of breast were included in this study. The patients were diagnosed clinically by specialist surgeon and they were among patients attending the radiotherapy and nuclear medicine hospital during the period of September 2015 through January 2017.

Control Group:

Twenty healthy individual who had no history or any clinical evidence of malignant disease. The control group's age and sex were matched with patient groups.

Investigation Carried Out During the Work:
1) Serum immunoglobulins Igs (IgG, IgA and IgM) level estimation by single radial immunodiffusion method (SRID).
2) Detection of CD4, CD8 and CD4/CD8 ratio using direct immunofluorescent test.

Estimation of Serum Immunoglobulins (IgG, IgA and IgM) Levels:
Quantitation of serum Igs of the study groups were carried out by SRID test:
1) Equal volumes (5μl) of the sample or control use human plasma as sample were added to wells in an agarose gel containing monospecific antisera.
2) The sample was diffused readily through the gel, the substances being assayed (antigens) form a precipitin ring with the monospecific antisera.
3) Ring diameters were measured following incubation at room temperature for 48 hrs and 72 hrs in case of IgM. Ring was calibrated using measuring viewer, and the reference curve is constructed on graph paper (standard Ig concentration versus squares of the rings diameters) unknown concentration were determined from the reference curve and expression as mg/dl.

Assessment of Specific Cell Mediated Immune Response:
Lymphocyte Separation:
The isopaque-ficol technique originally was used for lymphocyte separation.

Lymphocytes Counting and Viability Assessment:
Based on freshney[24], accurate numbers in a cell suspension can be calculated by counting the cells in a haemocytometer (e.g. improved neubauer) and trypan blue.

Slides Examination:
Slides were placed within the holder of the mechanical stage and the percent of cells were calculated by moving slide, in vertical examining pattern then in the horizontal examining pattern and the mean of the two reading was estimated.

Antisera and Kits and Media:
1) CD4 and CD8 kit (Dako A/S Denmark)
2) Single radial immune diffusion plate for accurate quantitative determination of human Igs (IgG, IgA and IgM) Biomegreb.

4. Results

Association of breast cancer patients ages and their percentage at different stages of the disease:
The ages of the female patients with malignant breast cancer included in this study ranged from 30-72 years with a mean age of (46.6 year). Table (1) shows the age distribution of different stages of the disease. Out of total 50 patients 6 were at stage I, 9 were at stage II, 16 at stage III and 19 at stage IV.

Healthy control group:
Twenty apparently healthy individual were randomly chosen. The healthy control ages were approximately matched with those of breast cancer patients group.

Table 1: Association of breast cancer patients ages and their percentage at different stages of the disease.

Age	Stage I	Stage II	Stage III	Stage IV	Total	%
20 – 29	0	0	0	0	0	0
30 – 39	2	5	4	0	11	22
40 – 49	1	3	7	7	18	36
50 – 59	2	1	5	8	16	32
60 – 69	1	0	0	3	4	8
70 – 79	0	0	0	1	1	2
Total	6	9	16	19	50	100

Immunoglobulins (Igs) levels:
Serum IgG level:
Figure 1 demonstrates the distribution of the IgG level in mg/dL of the healthy control group and that of patients with breast cancer of different stages of the disease. The mean IgG reading of the healthy control was (1231 mg/dL) with SD of $\pm$ 211. The mean IgG readings of patients at each stage of the disease and SD were as follow. At stage I was (1246 $\pm$ 220) at stage II was (1507 $\pm$ 237), at stage III was (1257 $\pm$ 417) and at stage IV was (1083 $\pm$ 216).
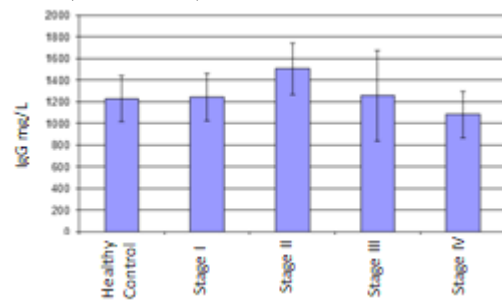

Figure 1: Distribution of IgG level in healthy control and patients with breast cancer at different stages of the disease (P = 0.008).

Serum IgM level:
Figure 2 shows the distribution of the IgM level in mg/dL of the healthy control group and that of patients with breast cancer at different stages of the disease. The mean reading of the healthy control was (135mg/dL) with SD of $\pm$ 41. The mean IgM reading of patient at each stage of the disease and SD was as follow. At stage I (141 $\pm$ 34), at stage II was (165 $\pm$ 56), at stage III was (126 $\pm$ 39) and at stage IV was (111 $\pm$ 36).

Serum IgA level:
Figure3 illustrate the distribution of the IgA level in mg/dL of the healthy control group and that of patients with breast cancer at different stages of the disease, the mean IgA reading of the healthy control was (278mg/dL) with SD of $\pm$ 78. The mean IgA readings of patients group at each stage and SD was as follow: At stage I was (235 $\pm$ 82), at stage II was (287 $\pm$ 99), at stage III was (264 $\pm$ 107), and at stage IV was (244 $\pm$ 135).
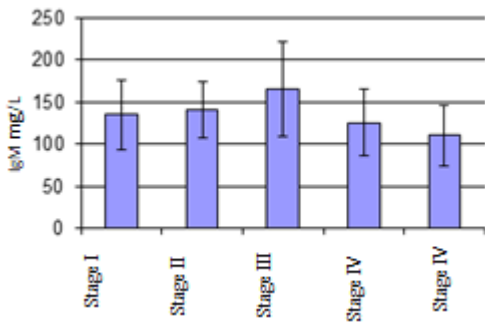
Figure 2: Distribution of IgM level in healthy control and patients with breast cancer at different stages of the disease (P = 0.029)
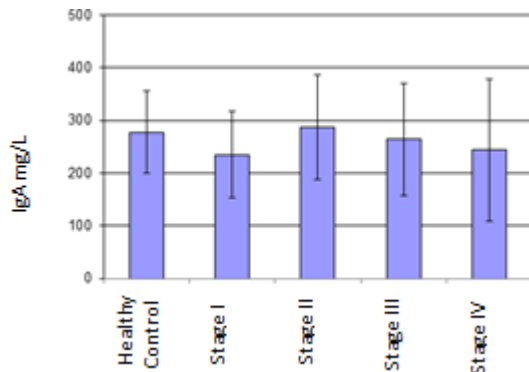

Figure 3: Distribution of IgA level in healthy control and patients with breast cancer at different stages of the disease (P = 0.755).

CD4 & CD8Markers:

CD4 Results:
Figure 4 shows the distribution of number of CD4 markers in healthy control and in patients with breast cancer at different stages of the disease. The mean CD4 count of the healthy control group was (37.911%) with SD of ± 5.310 while the mean reading at each stage of patient group and SD was as follows: At stage I group was (28.416 ± 4.978), at stage II was (26.988 ± 8.458), at stage III was (23.326 ± 3.899) and at stage IV was (17.510 ± 3.764).

CD8 Results:
Figure 5 demonstrate the distribution of number of CD8 marker of healthy control and patients with breast cancer at different stages of the disease. The mean count of the healthy control was (21.200%) with SD of ± 4.246 where was the mean reading of each stage and SD was as follow: At stage I was (21.983 ± 3.427) at stage II was (23.300 ± 2.263) at stage III was (23.900 ± 2.819) and finally at stage IV was (24.980 ± 2.417).

CD4/CD8 Ratio
Figure 6 illustrates the distribution of the ratio of CD4/CD8 markers in healthy control and that of patients with breast cancer at different stages of the disease.
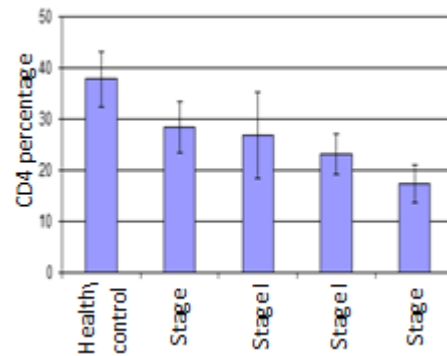

Figure 4: Distribution of CD4 in healthy control and in patients with breast cancer at different stages of the disease (P < 0.0001)
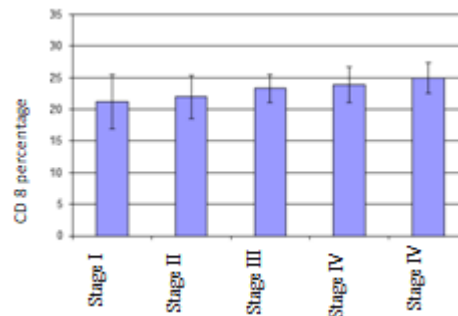

Figure 5: Distribution of CD8 in healthy control and in patients with breast cancer at different stages of the disease (P = 0.049).

The mean ratio of the healthy control was 1.788 with SD of ± 0.319 while the mean ratio of each stage and SD was as follows: At stage I was (1.299 ± 0.200), at stage II was (1.146 ± 0.282) at stage III was (0.976 ± 0.240) and at stage IV was (0.715 ± 0.201)
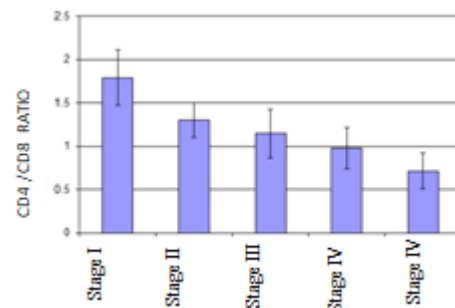.

Figure 6: Distribution of CD4/CD8in healthy control and in patients with breast cancer at different stages of the disease (P < 0.0001)

5. Discussion

Carcinoma of the breast is regarded as one of the common malignant tumors that occur frequently among old age group females, and it's incidence increased in frequency in the last few year.[5,6,16]

In this study the highest incidence of infection of breast cancer patients was at age 40-49 with a mean age of patients was 46.6 years. However younger age group and older age group are not exceptional. This incidence of breast cancer mean age is lower than that of American and European countries when the median age 62 years [5,25,26,27], and comparable to that of local studies reported by (Iraqi cancer board 2004) showing that the mean age was 49 years and to that of [28] who showed that the mean age of Iraqi breast cancer female patient was 51 years.

The incidence of carcinoma of breast in younger age Iraqi patients compared to other countries might be attributed to either environmental factors as uranium depleted, malnutrition, hormone change and stress or could be due to availability of newly introduced modern technological diagnostic procedures.

Immunoglobulins and Ab Production:
The characterization of immunoglobulin (Ig) level are essential for the diagnostic and therapeutic point of view.

The serum level of Igs in breast cancer patients in the present study group were quite close to their concentration in healthy control group, with slight decrease in the advanced stage and a significant increase in patients at stage II of the disease.

Patients with increased level of Igs could be due to over stimulation of B-cells which could occur at early stage of malignancy or it could be due to activation of B-cell by strong stimuli like immune complexes containing cross-linked Ag and complement, since such stimuli are more effective at B-cell activation than the Ag alone.

Decreased Igs level in advanced stage of breast cancer patients could be due to decrease in synthesis of the major Igs or blocked release of these Igs from B-cells, this in turn could be due to imbalance of T-lymphocyte subsets, in particular T-helper lymphocyte subset which help B-cell to produce Igs or it could be a result of complement depletion especially C3 and its splits product (ic_3b, c_3d) which lower the threshold for triggering B-cell activation, thus it plays an accessory, but not crucial rate in efficient induction of Abs response.[29]

CD4 and CD8 Markers &CD4/CD8 Ratio:
Tumor infiltrating lymphocytes, peripheral blood lymphocytes and lymph-node lymphocytes are the main effector cells to be studied.

Blood lymphocytes were isolated by density – gradient separation which relies on lymphocytes being less dense than erythrocytes and granulocytes, and immunofluorescence staining were used for their determination.

The present investigations of immunological features in patients with carcinoma of breast seem to be in favor of the hypothesis that impaired immunity and this impairment is revealed by: lymphopenia due to decrease of $CD4^+$ subpopulation of lymphocytes with the progress of the disease

reaching its lowest value at stage IV patients, which could be paralleled by defective Ab and cell mediated immune response, and by increased CD8 lymphocytes that fellow totally opposite reaction, being lowest at early stages of the disease reaching its highest level at stage IV, and consequently lead to decrease of $CD4^+$ / $CD8^+$ ratio which could be associated with a relative or constant increase in suppressor cells in patients group.

Lymphopenia could be a feature of protein – caloric malnutrition as suggested by Smythe et al., (1971)[30], or it could be due to stress induce immunosuppression which mediated by corticosteroids, endorphins and enkephalin which are all immunosuppressive in vivo. Steroid also prevent IFN gamma activation of APC, this in turn lead to in proper presentation to T-helper cell thus prevent their proliferation and differentiation. [29]

To compare the present results with other studies, it has been found that most of them were concerned with the tumor infiltrating lymphocytes, these studies showed that collection of tumor cells are extensively infiltrated by macrophages and $CD8^+$ (suppressor/ cytotoxic) T-cells, but by few $CD4^+$ (helper) T-cells.[12]

Baxevanis et al., (1994)[14] in their study of breast cancer patients found that CD4/CD8 ratio was 0.70. After expansion of lymphocyte with r/L-2 in the majority of patients, $CD3^+$ $CD8^+$ T-lymphocytes were present in greater number than $CD3^+$ $CD4^+$ T-lymphocytes. Recombinant IL-2 activated $CD3^+$ $CD8^+$ cells exhibited preferential cytolytic activity against autologous tumor cells.

A study on peripheral blood lymphocytes was done by Abelev (1989)[31], who suggested that cellular immunity was found suppressed in breast cancer patients and that there was a reversed ratio of CD4/CD8, low T-cell proliferation, and some immunity – suppressing factor in the peripheral blood. These abnormalities were most evident in advanced stages of breast cancer patients, result almost similar to the present finding. Moreover, it has been found that the ratio of CD4/CD8 increased significantly and the colony formation of T-cells greatly improved (5-6) months after radical mastectomy.

Bilik et al., (1989)[32], stated that in all the tumors studies there was a reversed CD4/CD8 ratio as compared with that found in normal peripheral blood. In more than half of the cases the CD4/CD8 ratio (helper/suppressor) was less than 1.0. The reversed ratio was due to a significant decrease in the number of helper cells. It is suggested that the reversed ratio of CD4/CD8 lymphocytes may significantly affect the host/tumor immune surveillance.

6. Conclusions

1) Breast cancer, though a common and fatal disease, is almost neglected, being diagnosed at advance stages of the disease in developing countries reflecting public ignorance of the importance of early diagnosis as well as

the lack of a well-planned program for screening and early detection.

2) In the present study the mean age of Iraqi women with breast cancer was (46.6) years, whereas the mean age of American women with breast cancer was (62) years. So it seems that the mean age of breast cancer patients varies in different geographical area environmental and other factors.

3) Number of CD4 positive cells decreased significantly with the progress of the disease, whereas number of CD8 positive cells increased with the progress of the disease.

4) Serum level of immunoglobulin increased significantly in early stages patient group compared to control group, but it's level decrease with advance stages.

7. Recommendation

1) Further study including large sample size of cases is needed to reveal specific link between immunological markers and pathology of carcinoma of breast before and after surgery in different stages of the disease.

2) Application of a suggested screening tests both laboratory and clinical for the early detection of breast cancer.

3) Evaluation of the level of different tumor markers before and after therapy and detecting their possible roles in predicting metastasis.

4) Further works are needed to clarify the role of different cytokines in manipulation of the immune response in breast cancer patients.

5) Studying the level of CD_4 and CD_8 cells in the tumor infiltrating with lymphocytes and in the lymph nodes and comparing its level with that of the serum.

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