Europ. J. Cancer Vol. 15, pp. 1491 1496
© Pergamon Press Ltd. 1979. Printed in Great Britain

0014-2964/79/1201-1491 $02.00/0

Leukocyte Adherence Inhibition (LAI) Tests in Patients Clinically Suspected of Having Breast Cancer Using a Panel of Breast Carcinoma Extracts

DIETER FRITZE,*† CHRISTIAN SCHULTE-UENTROP* and MANFRED KAUFMANN‡

*Medizinische Universitätsklinik, Heidelberg, Federal Republic of Germany and
‡Universitätsfrauenklinik, Heidelberg, Federal Republic of Germany

Abstract—*The question whether the LAI test could predict the histological presence or absence of breast cancer was investigated in this study. Fifty-six women hospitalized in the 'Frauenklinik' required a biopsy because clinical evidence suggested the diagnosis of breast cancer. Concurrently,* 34 *women were studied,* 14 *of whom were suffering from gynecological malignancies. Individuals were tested with several breast carcinoma extracts obtained from the panel available at that time. The extracts were prepared with* 3 mole/l *potassium chloride from the primary tumors of* 24 *different breast carcinomas. While all control subjects failed to respond to the panel of breast carcinoma extracts (mean LAI* $< 30\%$*),* 76% (29/38) *of the patients with confirmed breast cancer stage* I *or* II, 38% (3/8) *of the patients stage* III *or* IV *and* 40% (4/10) *of the patients with fibrocystic disease did respond to the panel of extracts. In LAI positive patients with fibrocystic disease, there was evidence of epithelial proliferation. Patients with benign and malignant breast lesions failed to react to one of the control extracts prepared from normal breast tissue or soft tissue sarcoma. Our results suggest that some patients with fibrocystic disease react similarly to patients with confirmed breast cancer in the LAI test, because their lymphocytes may have become sensitized to tissue antigens associated with breast carcinoma.*

INTRODUCTION

SEVERAL recent reports have suggested that the LAI test may detect specific tumor-associated immunity in a variety of human malignancies including carcinoma of the female breast [1–6]. In a few studies patients were tested prior to histological diagnosis [3–7]. In general, the majority of patients with benign breast lesions have been found not to react to breast carcinoma extracts in the LAI test [3, 4, 7]. However, some patients with fibrocystic disease have shown a reactivity similar to breast cancer patients [8]. In addition, soluble extracts derived from breast carcinoma tissue have been reported to inhibit the migration [9] and adherence [10] of lymphocytes from women with no clinical evidence of breast cancer who carried a substantial risk of developing this disease. Thus, a positive LAI response to breast carcinoma has been demonstrated in patients with confirmed malignancies as well as in some patients prone to developing breast cancer. In an attempt to validate further the LAI test in identifying patients prior to histological diagnosis, we have applied the procedure to a group of 'high risks' patients requiring surgery for diagnostic clarification.

Accepted 2 July 1979.

†Supported by grant SFB 136 from the Deutsche Forschungsgemeinschaft.

MATERIALS AND METHODS

Patients and controls

From June to December 1978, 56 women

aged 27–80 yr participated in this study. For all patients a biopsy was required, since physical examination and/or mammographic findings suggested the diagnosis of breast cancer. LAI was performed prior to histological diagnosis. At the same time, 34 women were studied to evaluate the reactivity of the breast carcinoma extracts. These controls, aged 21–75 yr, included 16 patients with one of the following malignancies: carcinoma of the ovary, (7); carcinoma of the corpus or collum uteri, (4); carcinoma of the vulva, (3); gastric cancer; (1); and non-Hodgkin's lymphoma, (1). Moreover, 4 patients with hypertension and/or diabetes, and 14 apparently healthy women (laboratory staff) were studied.

Extracts

Breast carcinoma extracts were prepared from the primary tumors of 24 patients with 3 mole/l potassium chloride by a modification [11] of the method reported by Meltzer *et al.* [12]. Control extracts were prepared from normal breast tissue and soft tissue sarcoma. The extracts were sterilized by Millipore® filtration. Their protein concentration, as determined by the Lowry method, ranged from 2.5 to 8 mg/ml. Individuals were tested with a panel of breast carcinoma extracts available at that time. An average of 6 breast carcinoma extracts were used to test the patients suspected of having breast cancer. In the control group an average of 5 extracts were used (Table 1). In addition, each patient was tested with one of the control extracts derived from soft tissue sarcoma or normal breast tissue.

LAI assay

LAI was determined in the wells of microtest II plates (Falcon, No. 3040) as previously described [13]. Briefly, blood lymphocytes were isolated according to the Böyum method [14] and suspended at 1×10^6/ml in Eagle's basal medium supplemented with 5% fetal calf serum (EBM). In most experiments, isolated blood lymphocytes were preincubated for 1 hr at 37°C in tissue culture flasks, after which the non-adherent cell fraction was used in the LAI test. This preincubation step was found to reduce non-specific suppressor cell-like activity mediated by B-cells and monocytes (unpublished observation). Effector cells then introduced into the LAI test contained 95% lymphocytes (mainly T-cells), less than 1% granulocytes and less than 5% monocytes, as determined by Wright's and esterase staining [15], and ingestion of latex particles [16]. One-hundred thousand lymphocytes in 0.25 ml EBM containing 75 μg tissue extract (final concentration 300 μg protein/ml) were incubated in each well of the microtest plates for 2 hr at 37°C. Then, the culture medium was aspirated from each well and the number of the non-adherent cells suspended in each well was counted in a Coulter counter. The mean percentage of adherent cells in triplicate cultures was determined, and LAI was calculated according to the formula:

$$\% \text{ LAI} = \frac{\% \text{ Adherence in EBM} - \% \text{ adherence in EBM plus extract}}{\% \text{ Adherence in EBM}} \times 100$$

For statistical analysis the chi-square test with Yates' correction was used taking the mean LAI value of 30% as the cut-off point.

RESULTS

In 46 of the 56 patients clinically suspected of having breast cancer, the diagnosis was confirmed histologically. Only 10 patients were found to be free of malignant disease.

The histogram of the LAI results, presented in Fig. 1, shows how often breast carcinoma extracts induced reactivity within LAI intervals of 10%. It may be seen that in the control group of 34 women the LAI reactivity tended towards a normal distribution between −15 and 25%. However, in the 46 patients with confirmed breast cancer, as well as in the 10 patients with fibrocystic disease, two similar curves of LAI distribution were obtained which showed a second peak between 30 and 50%. The histogram of LAI reactivity to a single control tissue extract, derived from soft tissue sarcoma or normal breast tissue, showed normal distribution between −5 and 25% (not shown).

Table 1 details that 4.9% (8/162) of the LAI results over 30% were obtained by reaction of lymphocytes from control subjects to breast carcinoma extracts. By contrast, 61% (193/318) of the LAI results results over 30% were obtained by reaction of lymphocytes from patients suspected of having breast cancer (Chi-square $\chi^2 = 135$, $P < 0.01$). Comparison by histology reveals that 66%

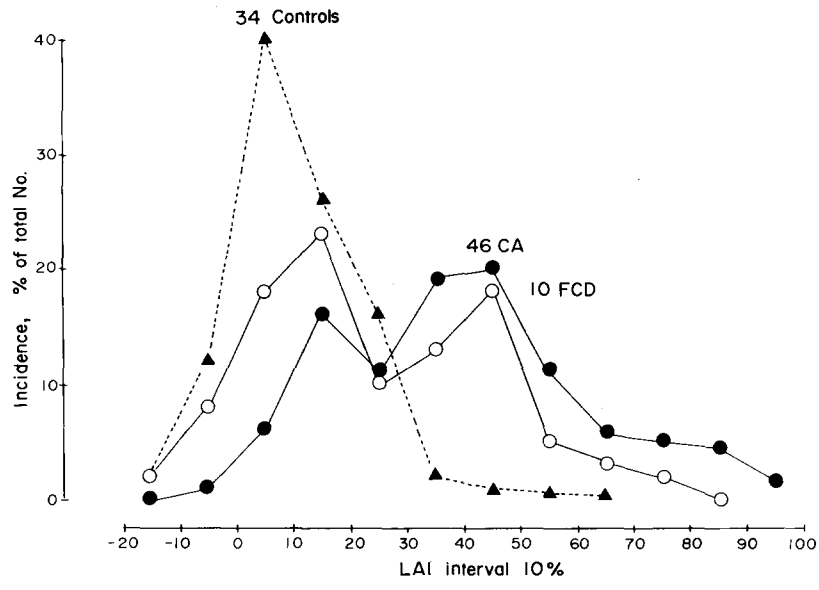

Fig. 1. Incidence (in % of total No.) of LAI reactions within intervals of 10% in 34 control subjects ▲----▲, 46 patients with confirmed breast cancer ●----●, and 10 patients with fibrocystic disease ○----○.

Table 1. Frequency of LAI results lower and higher than 30% in 56 patients suspected of having breast cancer and a control group of 34 women. (+ve/−ve = carcinoma confirmed or dismissed; control tissues were soft tissue sarcoma or normal breast tissue)

	Histology +ve/−ve	No. of LAI tests	Frequency of LAI <30%	Frequency of LAI >30%	Mean No. of extracts used	Type of extracts
Patients suspected of having breast cancer ($n=56$)	+	256	34%	66%	5.6	Breast–CA
	−	62	60%	40%	6.2	Breast–CA
	+/−	318	39%	61%	5.7	Breast–CA
	+/−	56	100%	0%	1.0	Control tissue
Control subjects ($n=34$)	Not done	162	95.1%	4.9%	4.8	Breast–CA

(168/256) of the LAI results over 30% were obtained with breast carcinoma extracts in patients with confirmed breast cancer, compared to 40% (25/62) in patients with fibrocystic disease ($\chi^2=12.3$, $P<0.01$). This difference was significant. Control extracts derived from soft tissue sarcoma and normal breast tissue failed to induce LAI over 30% reactivity in all patients studied.

We used a mean LAI value obtained with the panel of breast carcinoma extracts above and below 30% to call results positive or negative. The results, summarized in Table 2, show how often mean LAI values below 30%, between 30 and 50%, and over 50%, were obtained in patients and controls. While all control subjects failed to respond to the panel of breast carcinoma extracts (mean LAI <30% = LAI negative), positive LAI (mean LAI >30%) was noted in 76% (29/38) of the patients with confirmed breast cancer, stage I or II. A positive LAI was also noted for 38% (3/8) of the patients with confirmed stage III or IV breast cancer, and 40% (4/10) of the patients with fibrocystic disease. In the 4 LAI positive patients, fibrocystic disease was found to be associated with epithelial proliferation.

DISCUSSION

The results reported in this study indicate that 70% of the 46 patients with confirmed breast cancer reacted positively to a panel of

Table 2. Frequency of mean LAI results obtained with several breast carcinoma extracts in 56 patients suspected of having breast cancer (upper half) and 34 control subjects (lower half)

Disease (No. of patients)	Mean LAI (0–30%)	Mean LAI (31–50%)	Mean LAI (51–100%)	*n*
Breast cancer stage I/II	9	19	10	38
Breast cancer stage III/IV	5	2	1	8
Fibrocystic disease	6	4	0	10
Other malignant tumors	16	0	0	16
Hypertension/ diabetes	4	0	0	4
Healthy	14	0	0	14

breast carcinoma extracts, compared to 0% of the 34 controls ($P<0.01$). Other investigators reported results demonstrating a similar sensitivity in the LAI procedure [2–4, 7]. However, in most of the previous studies, lymphocytes of patients and controls were reacting to a single breast carcinoma extract rather than to a battery of extracts because the use of a panel of extracts has usually been found too tedious to be used for a routine testing. We chose a panel of breast carcinoma extracts in order to minimize experimental errors. Furthermore, we thought that breast cancer antigens are ill defined, and sensitization to these antigens might vary from one patient to another. Our results show that 76% of the patients with confirmed breast cancer stage I/II reacted to the panel of breast carcinoma extracts. This figure confirms the results reported from several other laboratories [1–7]. In the control group, 4.9% false positive LAI results were observed. (Table 1) These were probably due to an experimental error, because lymphocytes of the same donors did not respond to the other breast carcinoma extracts tested concurrently.

In this study, 40% (4/10) of the patients found to be free of malignant disease, showed a positive LAI. Only in those with positive results, was epithelial proliferation noted histologically. The data summarized in Table 3 indicate that these 'false positive' LAI results were obtained in a rather consistent fashion with up to 8 different breast carcinoma extracts. In 1 patient with fibrocystic disease, identical results were obtained in repeated experiments. Thus, an experimental error probably does not account for the 'false positive' LAI results in patients with fibrocystic disease associated with epithelial proliferation. We suggest that these patients reacted to the breast carcinoma extracts in a weaker manner (LAI, 30–50%) than a quarter of the patients with confirmed breast cancer (Table 2), because their lymphocytes had become less sensitized to (premalignant) antigens associated with breast carcinoma tissue.

It should be pointed out that we applied

Table 3. Results of LAI tests in patients with histologically confirmed fibrocystic disease. Patient 8 was tested twice

Patients	No. of extracts used	Adherence in EBM (%)	Mean LAI (%)	Range (%)	LAI +ve/−ve
1	7	67	0	−18–18	−
2	8	82	8	3–15	−
3	7	78	10	−3–27	−
4	7	81	24	9–43	−
5	7	74	19	−7–38	−
6	7	84	43	22–81	+
7	6	77	43	36–52	+
8a	8	73	49	36–68	+
8b	3	76	44	36–56	+
9	3	65	12	7–17	−
10	3	84	43	41–45	+

the LAI procedure to a selected group of patients not included in most previous reports. Our patients were admitted to the hospital because, in the opinion of the surgeon, the breast mass and/or mammographic findings were thought to be cancers rather than benign diseases. Therefore, 82% (46/56) of the patients suspected of having a breast cancer turned out to have the disease. We believe that in the 10 patients fortunately found to have a fibrocystic disease, the diagnosis of breast cancer could not be excluded by means other than biopsy. However, in previous reports published by Flores *et al.* [3] and Lopez *et al.* [7] more than half of the patients tested before surgery turned out to have benign breast lesions. Therefore, suspicion of breast cancer may have been considerably lower in their patients than in our patients who probably comprised a group of 'high risks'.

Flores *et al.* [3] noted that 54% of 509 patients admitted to the hospital with breast lumps were found to have benign lesions, of whom 10% showed positive LAI compared to 5% in the controls. Lopez *et al.* [7] reported that of 139 patients tested, 55% were found to have benign lesions, of whom 12% showed positive LAI compared to 3% in the controls. O'Connor *et al.* [8] concluded from a detailed analysis of false positive LAI tests in patients with benign breast lesions, that 63% (5/8) of the patients with intraductal atypia, not amounting to carcinoma *in situ*, had positive LAI results which were indistinguishable from those of breast cancer patients. Furthermore, Sanner *et al.* [10] reported positive LAI results in 24% of 161 patients with benign breast disease, of whom 50% (12/24) showed histological evidence for epithelial proliferation. Thus, in two previous reports as well as in our study, some patients with fibrocystic disease presented epithelial atypia and showed LAI results similar to breast cancer patients.

In mice [17] and patients with fibrocystic disease [18–20] the risk for developing a breast cancer is related to the grade of the epithelial proliferation. The expression of tumor antigens may precede the final mutational changes which then allow invasion and metastases. The results presented contribute to the current evidence that patients with proliferating fibrocystic disease and high risk of breast cancer might include a subgroup, recognized by a 'false positive' LAI response to breast carcinoma-associated antigens. On the other hand, our current experiments suggest that 30 out-patients from the mammography clinic show a low response rate (5%) to breast carcinoma extracts, which confirms the observation of Lopez *et al.* [7].

Several similarities between benign and malignant breast tissues have been reported. Dysplastic lesions of the mammary gland and breast cancer tissue have been found to share antigenic reactivity [21] and immunochemistry [22, 23], and may produce an 'angiogenesis factor' [24]. Further studies are planned to determine if patients with severe mammary dysplasia and positive LAI reactivity have a high risk of developing breast cancer.

Acknowledgements—The expert technical assistance of Miss Gerti Fedra is gratefully acknowledged.

REFERENCES

1. W. J. Halliday, Leukocyte adherence inhibition and blocking factors in cancer. In In Vitro *Methods in Cell-mediated and Tumor Immunity.* (Edited by B. R. Bloom and J. R. David) p. 547. Academic Press, New York (1976).
2. N. Grosser and D. M. P. Thomson, Cell-mediated antitumor immunity in breast cancer patients evaluated by antigen-induced leukocyte adherence inhibition in test tubes. *Cancer Res.* **35,** 2571 (1975).
3. M. Flores, J. H. Marti, N. Grosser, J. K. MacFarlane and D. M. P. Thomson, An overview: antitumor immunity in breast cancer assayed by tube leukocyte adherence inhibition. *Cancer (Philad.)* **39,** 494 (1977).
4. T. Fujisawa, S. R. Waldman and R. H. Yonemoto, Leukocyte adherence inhibition by soluble tumor antigens in breast cancer patients. *Cancer (Philad.)* **39,** 506 (1977).
5. D. Fritze, J. Fritze, M. Kaufmann and P. Drings, Immundiagnostische Aspekte beim Mammakarzinom. Das Phänomen der Leukozyten-Adhärenz-Inhibition. *Dtsch. med. Wschr.* **103,** 306 (1978).
6. D. Fritze, M. Kaufmann, P. Drings and G. Fedra, Selective inhibition of leukocyte adherence in women with breast cancer using a variety of tissue extracts. *Oncology* **35,** 242 (1978).

7. M. J. Lopez, R. O'Connor, J. K. MacFarlane and D. M. P. Thomson, Natural history of anti-tumor immunity in human breast cancer assayed by tube leukocyte adherence inhibition. *Brit. J. Cancer* **38,** 660 (1978).
8. R. O'Connor, J. K. MacFarlane, D. Murray and D. M. P. Thomson, A study of false positive and negative responses in the tube leukocyte adherence inhibition (tube LAI) assay. *Brit. J. Cancer* **38,** 674 (1978).
9. L. J. Brandes, M. K. Kiernan, G. Hardy, N. A. Nelson, G. J. Goldenberg, Correlation between leukocyte-migration inhibition by breast cancer antigens, mammographic findings and other breast cancer risk factors. *Lancet* **ii,** 626 (1977).
10. T. Sanner, I. Brennhovd, I. Christensen, O. Jorgensen and S. Kvaloy, Cellular antitumor immune response in women with risk factors for breast cancer. *Cancer Res.* **39,** 654 (1979).
11. J. A. Roth, H. K. Slocum, M. A. Pellegrino, E. C. Holmes and R. A. Reisfeld, Purification of soluble melanoma-associated antigens. *Cancer Res.* **36,** 2360 (1976).
12. M. S. Meltzer, E. J. Leonard, H. J. Rapp and T. Borsos, Tumor-specific antigen solubilized by hypertonic potassium chloride. *J. nat. Cancer Inst* **47,** 703 (1971).
13. D. Fritze, C. Schulte-Uentrop and M. Kaufmann, Leukocyte adherence inhibition test in breast cancer. *Lancet* **ii,** 742 (1978).
14. A. Böyum, Separation of blood leukocytes, granulocytes and lymphocytes. *Tiss. Antig.* **4,** 269 (1974).
15. L. T. Yam, C. Y. Li and W. H. Crosby, Cytochemical identification of monocytes and granulocytes. *Amer. J. clin. Path.* **55,** 283 (1971).
16. R. J. Winchester and G. Ross, Methods for enumerating lymphocyte populations. In *Manual of Clinical Immunology.* (Edited by N. R. Rose and H. Friedman) p. 64. American Society for Microbiology, Washington, D.C. (1976).
17. S. S. Brem, P. M. Gullino and D. Medina, Angiogenesis: a marker for neoplastic transformation of mammary papillary hyperplasia. *Science* **195,** 880 (1977).
18. P. K. Donnelly, K. W. Baker, J. A. Carnay and W. M. O'Fallon, Benign breast lesions and subsequent breast carcinoma in Rochester, Minnesota. *Mayo Clin. Proc.* **50,** 650 (1975).
19. R. R. Monson, S. Yen and B. MacMahon, Chronic mastitis and carcinoma of the breast. *Lancet* **ii,** 224 (1976).
20. D. L. Page, R. V. Zwaag, L. W. Rogers, L. T. Williams, W. E. Walker and W. H. Hartmann, Relation between component parts of fibrocystic disease complex and breast cancer. *J. nat. Cancer Inst.* **61,** 1055 (1978).
21. F. Avis, I. Avis, J. F. Newsome and G. Gaughiton, Antigenic cross-reactivity between adenocarcinoma of the breast and fibrocystic disease of the breast. *J. nat. Cancer Inst.* **56,** 17 (1976).
22. M. J. Lopez and D. M. P. Thomson, Isolation of breast cancer tumor antigen from serum and urine, *Int. J. Cancer* **20,** 834 (1977).
23. D. M. P. Thomson, D. N. Tataryn, R. O'Connor, J. Rauch, P. Friedlander, P. Gold and J. Shuster, Evidence for the expression of human tumor-specific antigens associated with β_2-microglobulin in human cancer and in some colon adenomas and benign breast lesions, *Cancer Res.* **39,** 604 (1979).
24. P. M. Gullino, Natural history of breast cancer. Progression from hyperplasia to neoplasia as predicted by angiogenesis. *Cancer (Philad.)* **39,** 2697 (1977).