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High endothelial venule and immunocompetent cells in typical medullary carcinoma of the breast

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Summary. The characteristics of immunocompetent cells and their role in killing tumour cells in typical medullary carcinoma of the breast (TMC) have been investigated morphologically. Formation of high endothelial venule (HEV)-like vessels in tumour cell nests, the distribution of macrophages, T-zone histiocytes, T- and B-lymphocytes, the ratios of CD4+/CD8+, and natural killer (NK) or NK-like T-cells were examined in five cases of TMC. These results were compared with controls which consisted of three cases of ductal carcinoma with intense lymphocytic infiltration (control I) and four cases of ductal carcinoma with scanty lymphocytic infiltration (control II). An increased incidence of HEV-like vessels with migration of lymphocytes and a higher number of CD8+ lymphocytes with interleukin-2-receptor expression, as well as numerous CD57 cells, were noted in the tumour nests of TMC as compared with those of control groups. Furthermore, large granular lymphocytes, large lymphocytes invaginating tumour cells and necrotic tumour cells were observed electron microscopically. These findings indicate that infiltrating lymphocytes in TMC are activated and become effector cells that can kill the tumour cells by mechanisms similar to those of NK cells. The activities of immunocompetent cells in TMC appear to contribute to a favourable prognosis in TMC of the breast.

Introduction

It is generally accepted that medullary carcinoma of the breast has a more favourable prognosis than other histological types of breast carcinoma (Rindolfi et al. 1977). Rapin et al. (1988) reported that the 10-year disease-free survival rate of patients with typical medullary carcinoma (TMC) was 92% compared with 52% for patients with non-medullary carcinoma. Although one of the characteristics of TMC is conspicuous lymphocytic infiltration, the functions of these lymphocytes have not been clarified. We previously reported that lymphocytes infiltrating a ductal carcinoma of the breast did not appear to be activated or to be effector cells in the killing of tumour cells (Tanaka et al. 1986). In contrast, it had also been noted, in the earlier study, that numerous T-cells in TMC tumour nests expressed interleukin-2 (IL-2) receptor and were thought to be activated T-cells. The infiltrating lymphocytes in TMC are suspected to differ functionally from those in ductal carcinoma and to have the ability to kill tumour cells. For the purpose of clarifying the function of TMC lymphocytes, we have now performed immunohistochemical and electron microscopical investigations.

This study revealed that lymphocytes infiltrating TMC migrated through high endothelial venule (HEV)-like vessels in tumour cell nests were activated in situ and then became effector cells and attacked tumour cells. The characteristics of these cells in TMC and ductal carcinoma are compared and discussed.

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Materials and methods

Surgically resected primary breast carcinoma tissues were used in this study. Five cases which had been pathologically diagnosed as TMC (according to the classification of Rapin) were studied. Three cases of ductal carcinoma accompanied by severe lymphocytic infiltration (control I) and four cases of ductal carcinoma with mild lymphocytic infiltration (control II) served as controls. The patients' ages ranged from 29 to 58 years in TMC cases, 47–65 years in control I and 37–55 years in control II. One part of each specimen was snap frozen and serial cryostat sections were made. The other was fixed in formalin and embedded in paraffin wax for serial sectioning.

Fresh frozen sections were fixed in acetone for 15 min at room temperature and paraffin sections were dewaxed, and then the sections were submitted to the following immunostainings after washing with 0.01 M phosphate buffered saline (PBS), pH 7.4. For detection of CD4, CD8, CD19, Tac, CD71, CD57, CD56 and CD68 (Table 1), the ABC method (avidin-biotin-peroxidase complex;

Table 1. Immunohistochemical methods of antigen detection

Antigens	Antibody	Dilution of antibody	Supplier	Method of detection
Fresh frozen section				
CD4	Anti-Leu3	1:40	Becton Dickinson (San Jose, CA, USA)	
CD8	Anti-Leu2	1:80	Becton Dickinson	
CD19	Anti-pan B	1:50	Dakopatts, Glostrup, Denmark	ABC
IL-2 receptor	Anti-Tac	1:3000	Dr. Uchiyama, Kyoto Univ.	(Vector, Burlingame, Calif.)
CD71	OKT9	1:50	Orth (Raritan, NJ, USA)	
CD16	Anti-Leu11	1:25	Becton Dickinson	
CD57	Anti-Leu7	1:50	Becton Dickinson	
CD56	Anti-Leu19	1:50	Becton Dickinson	
Paraffin section				
CD68	KP1	1:50	Dakopatts	ABC (Vector)
S100 protein	Anti-S100 protein	1:200	Dakopatts	PAP
Factor VIII	Anti-factor VIII	1:500	Dakopatts	(Dakopatts)

Vectastain ABC Kit, Vector, Burlingame, Calif., USA) was used. After incubation in normal horse serum for 10 min at room temperature, the frozen sections were treated with the primary monoclonal antibody at 4° C overnight. Normal mouse serum was used instead of the primary antibodies for staining control. After washing with PBS, the sections were treated with biotinylated anti-mouse IgG horse IgG for 30 min at room temperature, and then were incubated in ABC for 60 min at room temperature. The slides were washed with PBS and reacted with 0.05% 3,3′-diaminobenzidine tetrahydrochloride (DAB) and 0.01% hydrogen peroxide/0.05 M TRIS-HCl buffer, pH 7.6 for 3–5 min.

In order to block endogenous peroxidase, dewaxed and rehydrated sections were incubated in 0.3% hydrogen peroxide/methanol for 30 min at room temperature, and then washed in PBS. After incubation in 10% normal goat serum for 10 min at room temperature, sections were reacted with the primary antibody at 4° C overnight. For staining control, normal rabbit serum was applied instead of the primary antibodies. After washing with PBS, the sections were treated with anti-rabbit IgG goat serum (1:200) for 30 min at 37° C, and then incubated in peroxidase-anti-peroxidase complex (Dakopatts, Glostrup, Denmark) (1:100) for 30 min at 37° C. Detection of the reaction products was carried out using 0.02% DAB and 0.005% hydrogen peroxide. Sections were counterstained with haematoxylin.

Quantitative analysis of tumour-associated CD4+, CD8+, and CD57+ lymphocytes was done using a micrometer. The number of lymphocytes per 250 μm^2 was counted by high power view microscopy from 15 to 25 fields of each tumour cell nest, and the mean value was calculated.

For the electron microscopic study specimens were fixed in 2% glutaraldehyde either with or without 0.1% tannic acid, post-fixed in 1% osmium tetroxide, followed by dehydration and embedding in epoxy resin. Ultra-thin sections were doubly stained with uranyl acetate and lead citrate, and examined using a Hitachi H-7000 electron microscope.

The maximum diameter of lymphocytes was measured on the photographs.

Results

Although B-lymphocytes were abundant in the follicle-like structure outside tumour cell nests, there were few B-lymphocytes within the TMC tumour (Fig. 1a, b). One of five cases of TMC showed only slight B-cell infiltration. However, B-lymphocytes were frequently found in all cases of ductal carcinoma with intensive lymphocytic infiltration (Fig. 1c, d). T-lymphocytes were the main population in TMC tumour nests and in controls I and II. The density of T-lymphocytes in the tumour nests was higher in all cases of TMC than in control cases.

The distributions of CD4+ and CD8+ lymphocytes did not differ among any of the cases. Ratios of CD4+/CD8+ lymphocytes, however, were significantly lower in TMC (0.48+0.07) than in control II (0.72+0.15) or in control I (1.11+0.15) (Table 2). This indicated a tendency for fewer CD4+ lymphocytes to be present in TMC than in control cases.

IL-2 receptor (Tac; Uchiyama et al. 1981) and transferrin receptor (CD71) were used as indicators of activation of T-lymphocytes. Tac antigen was observed on most of the lymphocytes in the tumour cell nests in two of five cases (Fig. 2a) and in approximately half of the lymphocytes in the other cases of TMC. CD71 antigen was also demonstrated in TMC, but on fewer lymphocytes than the Tac antigen. Neither antigen could be found in tumour nests of control I, but a few positive lymphocytes were noted in peripheral foci of dense lymphocytic aggregation (Fig. 2b). There were a few Tac-positive or CD71-positive lymphocytes in control II.

Macrophages are indispensable to the lymphokine cascade for lymphocyte activation and macrophages were thus detected with KP1 monoclonal antibody (CD68) and T-zone histiocytes were demonstrated by localization of S100 protein (Furukawa et al. 1985) (Table 2). CD68+ macrophages were scattered in tumour cell nests of every group at similar grades. There was no tendency for a greater number of CD68+ cells to be present in TMC than in controls. S100-protein-positive histiocytes were rarely seen in control II but were found in two cases of control I. There were many S100-protein-positive histiocytes in two cases of TMC, and the other cases of TMC showed a small number. These cells were scattered within foci of infiltrating lymphocytes in the tumour nests.

Localization of natural killer (NK) cells was revealed by anti-CD16. The NK cell population containing NK-like T-cells was shown by anti-CD57. Anti-CD56 antibody revealed NK-cells and a subset of T-lymphocytes that mediated MHC-unrestricted cell-mediated cytotoxicity (Grossman and Herberman 1986; Lanier et al. 1987;

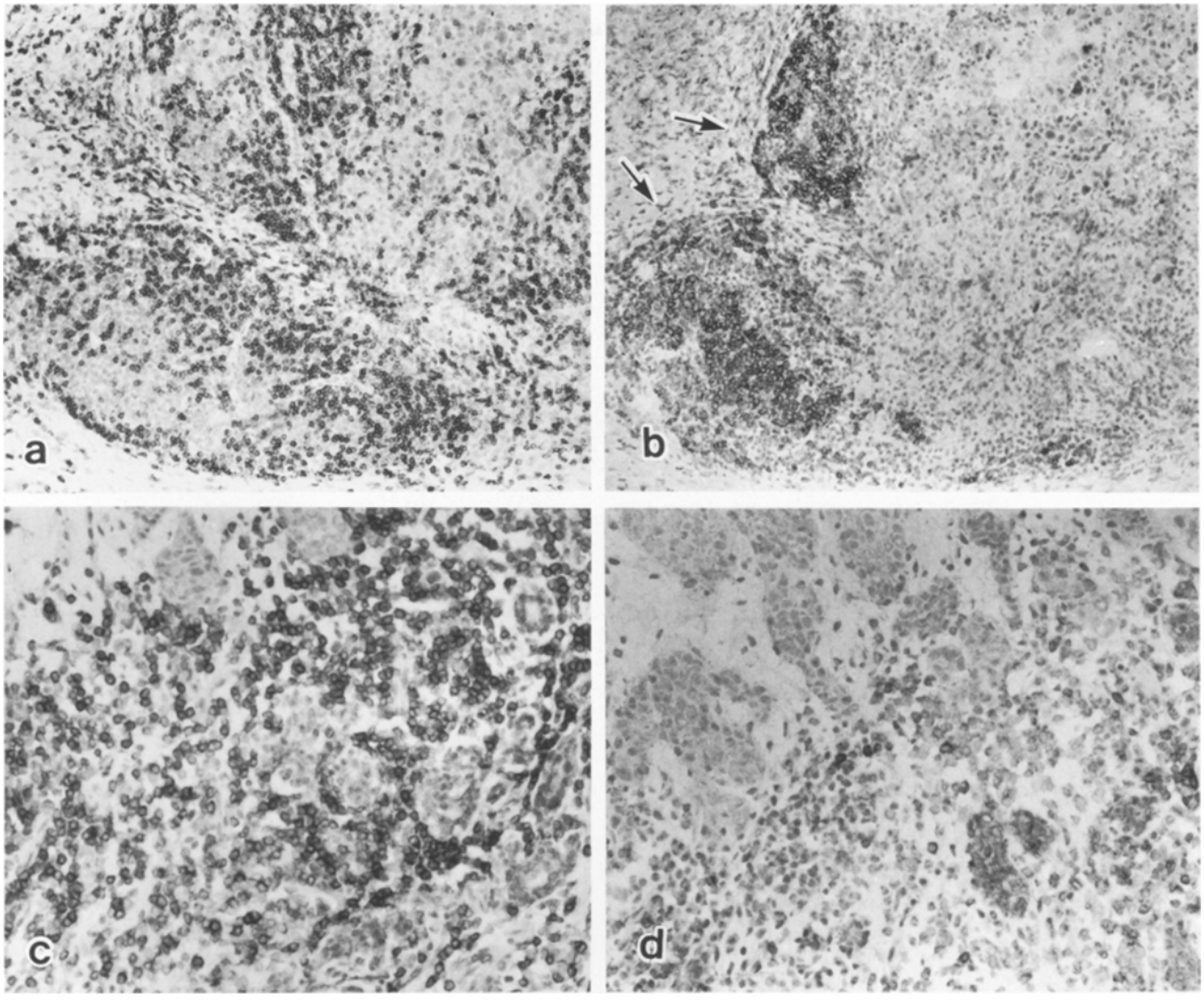

Fig. 1. Distribution of T- and B-lymphocytes is shown immunohistochemically in typical medullary carcinoma (TMC) **a**, **b** and in control I **c**, **d**. In TMC, B-lymphocytes aggregate in follicle-like structures (*arrow*) outside tumour cell nests (**b**: left one-third), whereas T-lymphocytes **a** are located around this structure and scattered in tumour cell nests (**a**: right upper). Abundant T-lymphocytes **c** and a small number of B-lymphocytes **d** are noted around tumour cell nest of control I. a, b $\times 25$; c, d $\times 50$

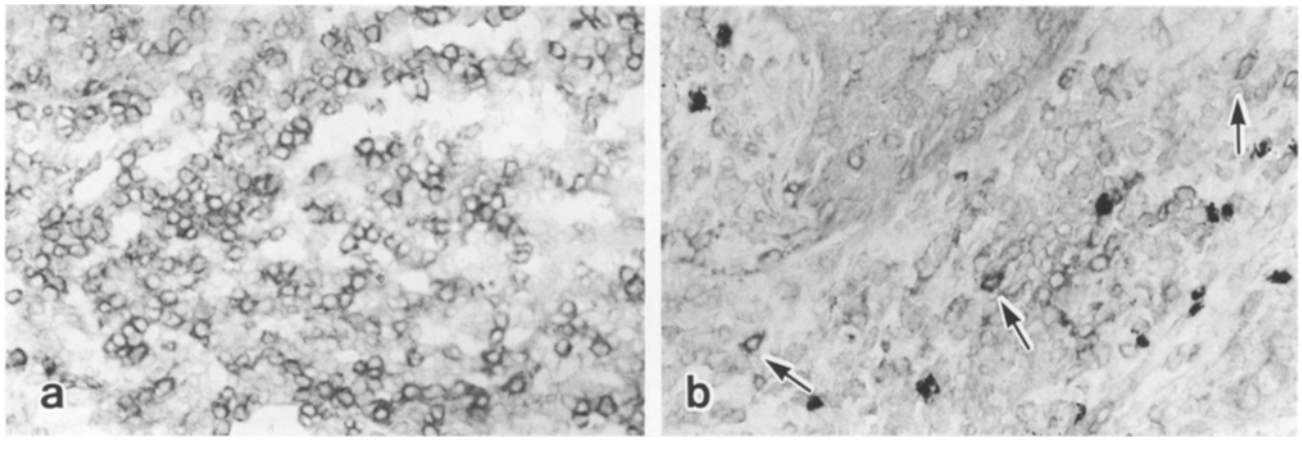

Fig. 2. Tac-positive cells in TMC **a** are numerous, but they are scattered in control I (**b**, *arrows*). Counterstained with haematoxylin, $\times 80$

Table 2. Summary of immunohistochemical and ultrastructural findings

	Typical medullary carcinoma					Control I[a]			Control II[b]			
Case no. (age)	18 (58)	42 (44)	116 (56)	124 (55)	131 (29)	67 (47)	76 (55)	130 (66)	61 (47)	117 (55)	125 (39)	127 (47)
Tumour nest lymphocytes												
T-lymphocyte	+++	+++	+++	+++	+++	+	++	+++	+	+	+	±
B-lymphocyte	−	±	−	−	+	+	±	+	±	±	+	−
CD4+/CD8+	0.48	0.48	0.50	0.58	0.45	1.49	0.97	1.27	0.71	0.93	0.59	0.63
Tac+lymphocyte	+++	++	++	+++	++	+	±	+	+	±	±	−
CD71+lymphocyte	++	+	+	+	+	+	±	±	±	±	±	±
CD16+lymphocyte	±	±	±	±	±	−	−	−	−	−	−	±
CD57+lymphocyte/ 250 μm²	33.7	5.0	14.7	24.3	2.0	0.9	0.8	7.8	0.8	7.4	0.9	0.8
CD56+lymphocyte	ND	ND	±	+	±	ND	ND	±	ND	±	+	±
Macrophage												
CD68+	+++	+++	+++	+++	+++	+++	+	+++	++	+++	+++	+++
S100 protein+	+	+++	+	+++	+	+−+++	±	+	−	−	±	±
High endothelial venule	+++	+++	+++	++++	+++	++	++	++	−	−	±	−
Max. diameter of lymphocyte (μm)	ND	ND	ND	8.5 ±1.2	8.0 ±1.8	ND	ND	ND	ND	6.1 ±1.1	5.5 ±1.0	5.6 ±1.2
Invagination of lymphocyte into tumour cell	ND	ND	ND	++	++	ND	ND	ND	ND	ND	−	−

[a] Ductal carcinoma with intense lymphocytic infiltration
[b] Ductal carcinoma with scanty lymphocytic infiltration
± ~ +++; Number of positive cells. ±, very small; +, small; ++, large; +++, very large; ND, not determined

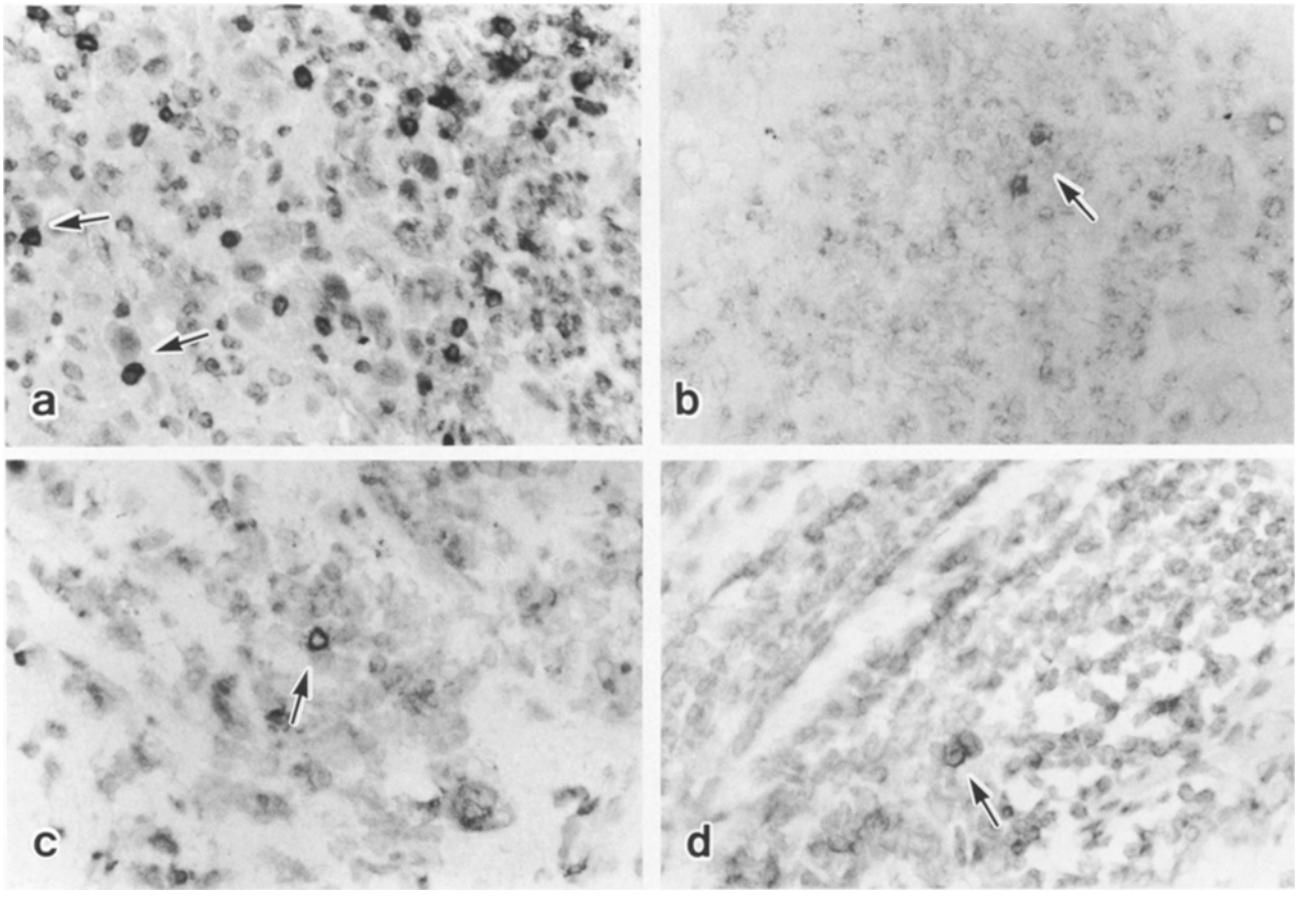

Fig. 3. CD57-positive cells **a**, **c** and CD56-positive cells **b**, **d** in TMC **a**, **b** and control I **c**, **d**. Many CD57-positive cells scattered in tumour cell nest of TMC and some in contact with tumour cells (**a**, *arrow*). While a small number of CD56-positive cells can be found in it (**b**, *arrow*), CD57- or CD56-positive cells are not seen in control I **c**, **d**, respectively. Counterstained with haematoxylin, ×100

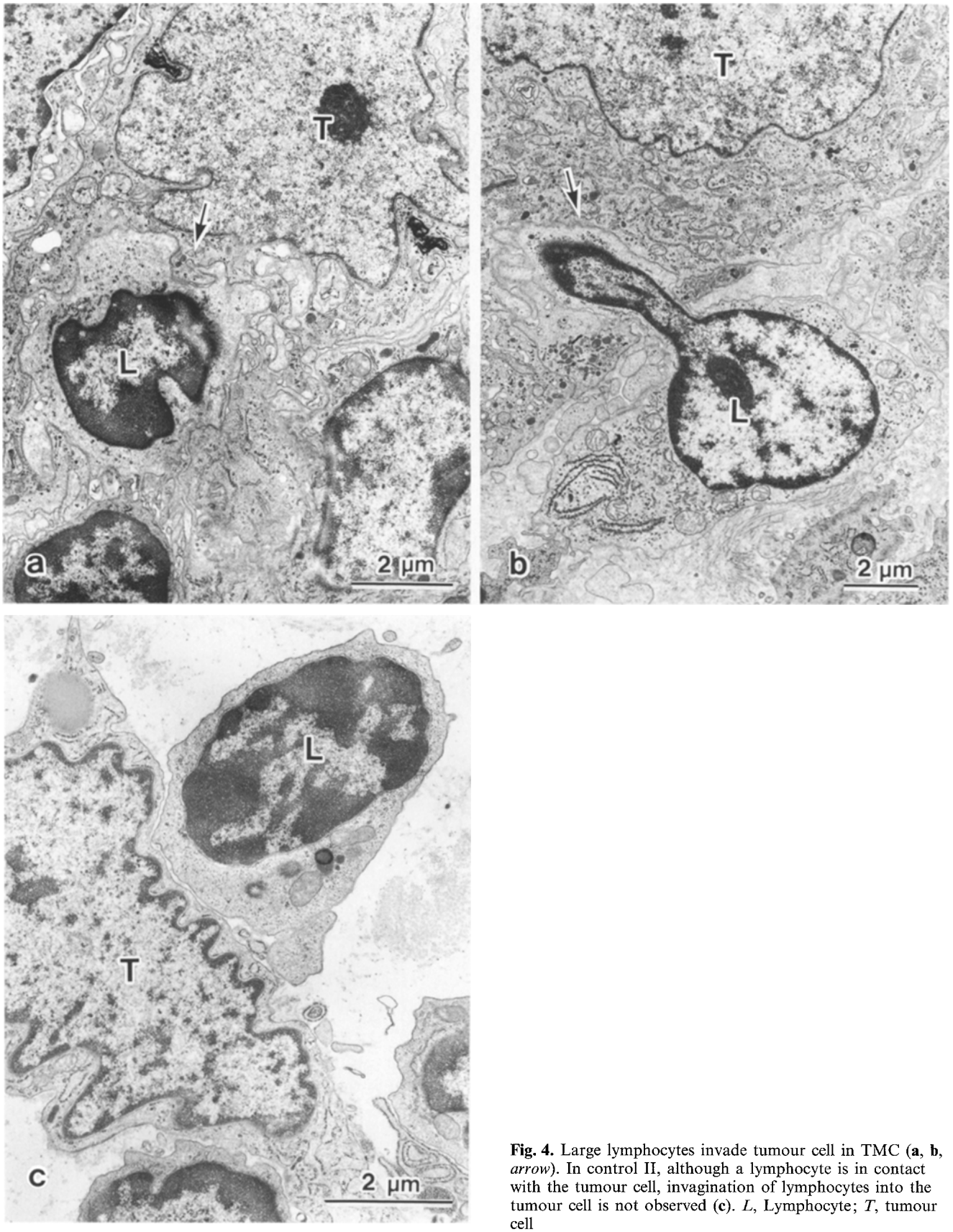

Fig. 4. Large lymphocytes invade tumour cell in TMC (**a**, **b**, *arrow*). In control II, although a lymphocyte is in contact with the tumour cell, invagination of lymphocytes into the tumour cell is not observed (**c**). *L*, Lymphocyte; *T*, tumour cell

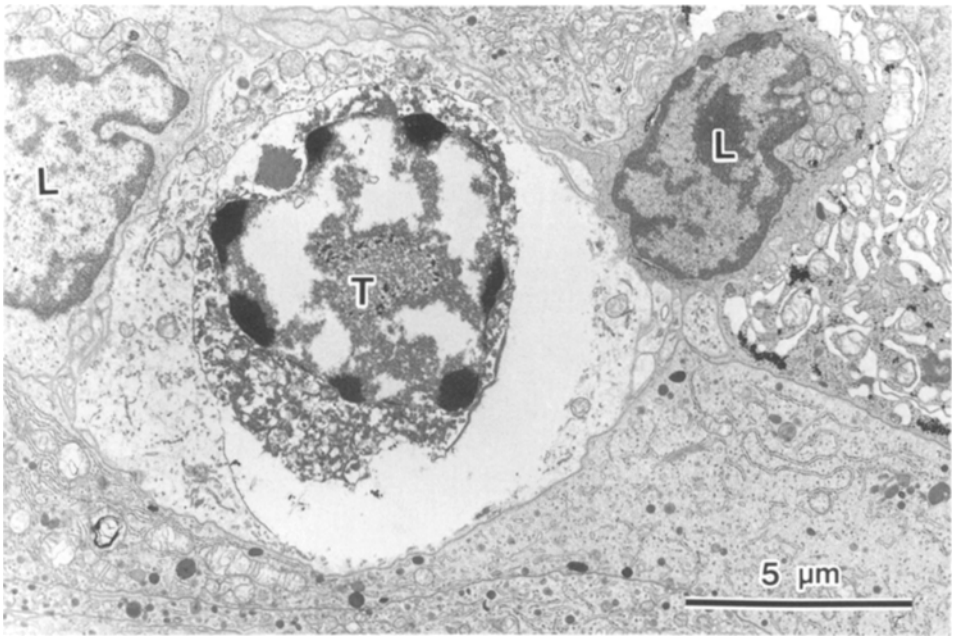

Fig. 5. Necrosis of a tumour cell with two attached lymphocytes in TMC. *L*, lymphocyte; *T*, necrotic tumour cell

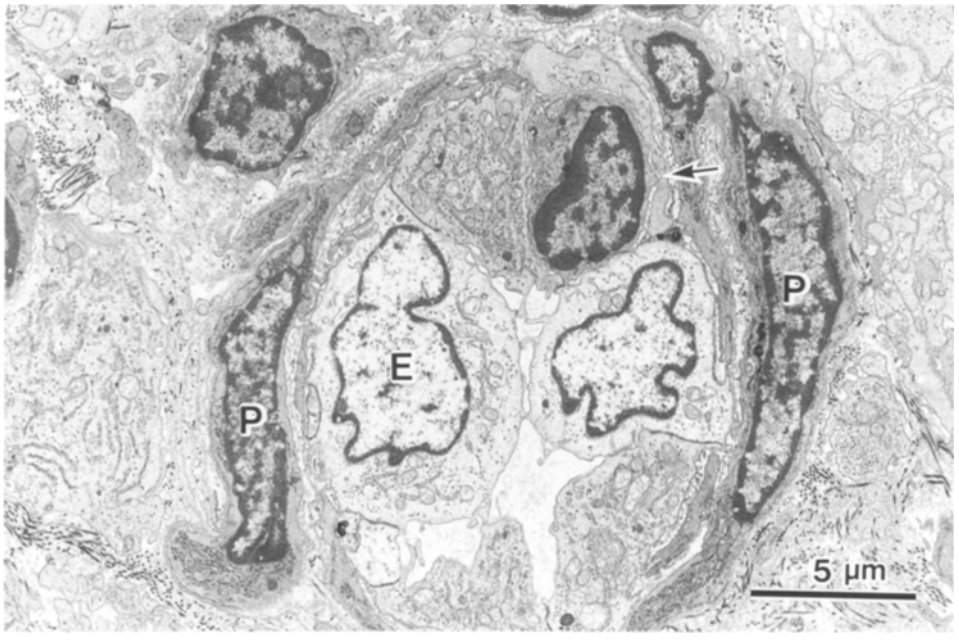

Fig. 6. Note high endothelial venule (HEV)-like vessel and lymphocyte migration between endothelial cells (*arrow*) in TMC. *E*, Endothelial cell; *P*, pericyte

Rabinowich et al. 1987; Ritz et al. 1988). Small numbers of CD16+ cells were observed in follicle-like structures localized around the TMC tumour nests but they were not found in either control I or II. CD57+ cells were frequently noted in TMC tumour nests (Fig. 3a) and a small number was also seen in peripheral areas of nests in control I (Fig. 3c). CD56+ cells were rarely noted in control groups (Fig. 3d), but in TMC, CD56+ cells were occasionally observed and they were also found in the tumour cell nests (Fig. 3b).

Ultrastructural examination was performed in two cases of TMC and two cases of control II. In general, there were some regions consisting of light and dark tumour cells and others showing infiltration of immunocompetent cells mixed with tumour cells.

Although the lymphocytes infiltrating TMC were of various sizes, there was a tendency for these lymphocytes to be larger in TMC in comparison with those seen in controls (Table 2). They were frequently in contact with a macrophage or an interdigitating cell (T-zone histiocyte) and interdigitations of the microvilli of both cells were noted. The lymphocytes in contact with tumour cells were generally large, having large quantities of cytoplasm with well-developed organelles, and sometimes granules. In a small number, the microvilli or part of the lymphocyte invaginated into the tumour cells

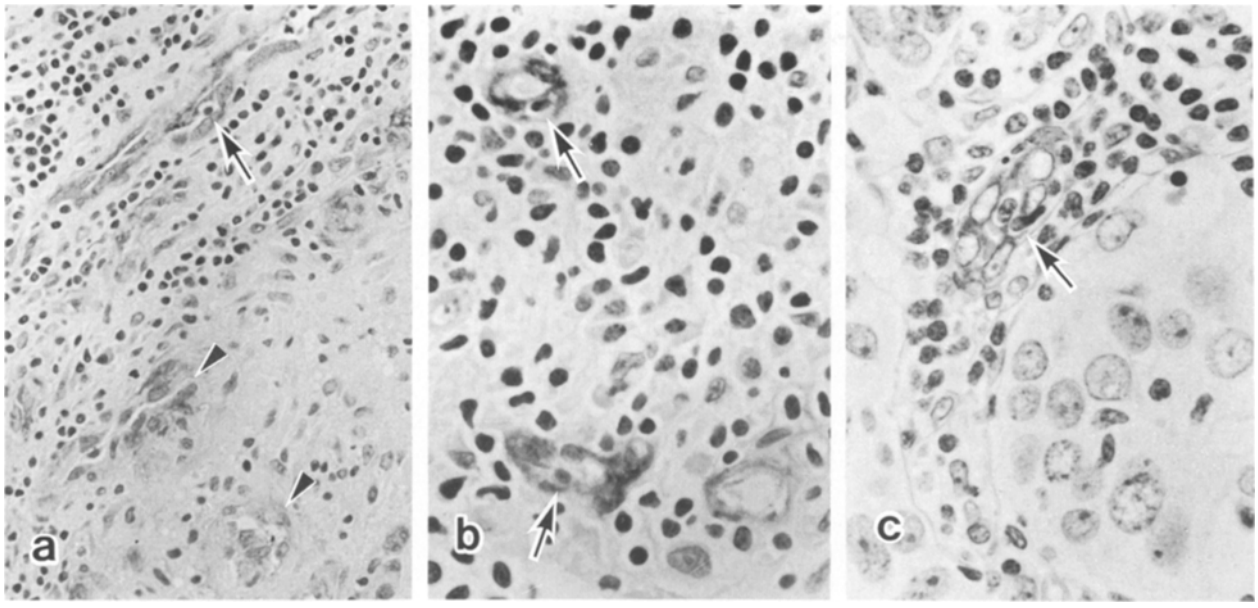

Fig. 7. HEV-like vessels revealed by localization of factor VIII (**a**, **b** TMC; **c** control I). Many factor-VIII-positive HEV-like vessels are noted not only in the foci with lymphocytic infiltration (upper left of **a**) but also in tumour cell nests (lower right of **a**, **b**; *arrowhead*). Migration of lymphocytes through endothelium is observed (*arrow*). An HEV-like vessel with lymphocyte migration (*arrow*) is found only in the lymphocytic infiltrated foci of control I (**c**). Counterstained with haematoxylin; **a** ×80; **b**, **c** ×160

(Fig. 4a, b). This invagination was not associated with disruption of the tumour cell membrane, and the membranes of both cells were preserved at the site of lymphocyte invagination. Degeneration or necrosis of those tumour cells in contact with lymphocytes was found occasionally in TMC (Fig. 5). In controls although middle-sized lymphocytes were occassionally observed to be in contact with the tumour cell membrane, no invagination of the lymphocyte into the tumour cell could be found (Fig. 4c).

HEV-like vessels were frequently noted in foci of lymphocytic infiltration in TMC, and it appeared that lymphocytes had migrated through the endothelium (Fig. 6).

Since HEV-like vessels were identified electron microscopically in TMC, we sought to determine whether HEV-like vessels were specific for TMC using antibody for factor VIII in paraffin-embedded sections of TMC and controls I and II.

HEV-like vessels were observed frequently in lymphocyte-infiltrated foci in TMC (Fig. 7a, b) and control I (Fig. 7c). HEV-like vessels were more abundant in the TMC tumour nests than in control I. There were no factor-VIII-positive HEV-like vessels in control II, which also had a little lymphocyte infiltration.

Discussion

It has been reported that a favourable prognosis might be expected in tumours with intense lymphocytic infiltration (Pederson et al. 1988; Rapin et al. 1988; Rindolfi et al. 1977; Rosen et al. 1977; Wargotz and Silverberg 1988). However, the underlying mechanisms have not been clarified.

Recently, attempts have been made to activate isolated tumour-infiltrating lymphocytes (TIL) with IL2 so as to create effector cells targeted against the tumour cells (Belldegrum et al. 1988; Heo et al. 1987; Itoh et al. 1986; Muul et al. 1987; Shau et al. 1988; Topalian et al. 1989). TIL have been reported to be more effective than lymphokine activated killer cells for killing tumour cells in vivo (Rosenberg et al. 1986) and these facts suggest that immunocompetent cells, including lymphocytes, may play a role in vivo in killing of tumour cells. We have reported, however, that the lymphocytes that infiltrate ductal carcinoma were not cytotoxic for tumour cells (Tanaka et al. 1986). However, the high frequency of activated T-cells in TMC tumour, in contrast to other histological types of breast carcinoma, suggested to us that the lymphocytes could be the effectors in the killing of tumour cells, and thus could contribute to the favourable prognosis.

In this study, five cases of TMC were examined and we were consequently able to identify certain differences between TMC and ductal carcinoma, with or without lymphocyte infiltration (control I or control II respectively) as shown in Table 2.

HEV-like vessels were observed in TMC and control I, and the lymphocytes were thought to have migrated with ease into the tumour tissue. HEV-like vessels have been reported in medullary carcinoma of the breast (Ahmed 1980) and in foci with lymphocytic infiltration around malignant neoplasms (Freemont 1982). Our study also revealed factor-VIII-positive HEV-like vessels in tumour cell nests. A higher frequency of HEV-like vessels was observed in TMC than that in control I, while these vessels could not be found in control II. Since stimulation with antigens, interferon, tumour ne-

crosis factor or IL-1 have been reported to correlate with formation HEV (Cavender et al. 1987; Manolios et al. 1988), it is possible that the endothelium of the post-capillary venule in the tumour foci of TMC or control I received such a stimulus, and transformed into HEV that allowed the migration of lymphocytes into the tumour foci.

Localization of macrophages, which are essential for activation of lymphocytes and formation of HEV, was demonstrated by anti-CD68 monoclonal antibody. Many CD68+ macrophages were noted even in control II, in which lymphocytes were scarce. This finding might imply that the circumstances necessary for formation of HEV are already present in every histological type of breast carcinoma. However, the reactions of the endothelium to stimuli induced by macrophages were different depending on the histological type of cancer.

Among the lymphocytes infiltrating TMC tumour nests, the CD8+ subset population was higher and that expression of IL-2 receptor was more frequent than in controls I and II. Furthermore, though CD16+ and CD56+ cells were scarce, the populations of CD57+ lymphocyte were higher in TMC. We speculate that TMC lymphocytes migrating through HEV-like vessels are activated and become effector cells for the killing of the tumour cells. Ultrastructural findings that the lymphocytes that infiltrate TMC are larger than those in ductal carcinoma, frequently have microvilli and occassionally invade tumour cells seem to support our speculation. It has previously been reported that IL-2-stimulated TIL invaded and killed tumour cells in vitro (Tanaka et al. 1991). Close similarity between the ultrastructural characteristics of these lymphocytes in TMC and the previously described TIL suggests that effector lymphocytes that can kill tumour cells exist in the TMC tumour cell nests.

The granules in TIL have been suggested to participate in the cytotoxic effects (Iwasaki et al. 1990; Lowrey et al. 1988; Tanaka et al. 1991) and the role of NK-like T-cell and NK cell in killing tumour cells cells in vitro has been discussed (Heo et al. 1988; Shau et al. 1988). In TMC, granules were noted in large lymphocytes. Furthermore, lack of MHC class I expression in the plasma membranes of TMC tumour cells has been reported (Tanaka et al. 1987). These observations indicate that cytotoxic T-lymphocytes could not kill tumour cells in TMC by class I MHC restriction, but it is possible that NK-like T-cells might play a role in killing tumour cells as effector cells, as in IL-2-stimulated TIL.

The question remains as to why the lymphocytes that infiltrate control I could not be activated. Macrophage infiltrations in control I and TMC were of similar grade, and a higher number of CD4+ lymphocytes was noted in the former. It is possible that the lymphokine cascade was activated more easily in control I than in TMC. The data from our studies are insufficient to provide an answer to this question. The microenvironment of the cancer cell nest is considered to be an important factor in stimulating immune reactions. Several kinds of T-cell suppressor factors from human tumour cells have been reported (Hersy et al. 1983; Martin et al. 1987; Roth et al. 1983; Serrano et al. 1990; Wrann et al. 1987). The factors influencing the microenvironment of breast carcinoma should be investigated and the possibility that production of a T-cell suppressor factor is lower in TMC than in other histological types of breast carcinoma should be investigated further.

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