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LYMPHOID CELLS IN CARCINOMA OF THE BREAST

Failure of response to phytohaemagglutinin in vitro

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The peripheral lymphoid organs of vertebrates contain two major classes of immunocompetent lymphoid cells. One of these is dependent on the thymus (T-cells) and the other on the bursa of Fabricius in avians (B-cells) (ROITT et coll. 1969) and its possible analogue in mammals (COOPER et coll. 1966). Investigations into the survival of allogenic and xenogenic grafts in thymectomized individuals indicate that T-cells are necessary for graft nonacceptance (MILLER & OSOBA 1967). Experimental support also exists for the view that the presence of T-cells is important for the rejection of certain syngenic tumours (MILLER et coll. 1964, GRANT et coll. 1965). The role of B-cells during graft rejection is less clear. Experimental data indicate that thymus-independent lymphocytes may lyse cells in vitro, being coated with small amounts of antibodies directed against specific membrane antigens (HARDIN et coll. 1971, BOXEL et coll. 1972).

The prognosis is considered to be favourable in different types of malignant growths with histologic evidence of infiltration of lymphocytes (HULTBORN & TÖRNBERG 1960, CUTLER et coll. 1969). The invasion may be interpreted as a sign of active immunological reaction against the tumour. The present in-

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vestigation is an attempt to characterise the lymphocytes infiltrating carcinoma of the breast as regards their capacity to be activated by phytohaemagglutinin in vitro.

Methods. The primary diagnosis of mammary carcinoma is usually made by fine needle aspiration biopsy (FRANZÉN & ZAJICEK 1968). Four consecutive cases with cytologic signs of extensive lymphocyte infiltration in the tumour were selected. A piece of the tumour was dissected out within 30 minutes of removal of the breast. Cells from the neoplasms were obtained by gently cutting them into thin slices in Eagle's minimal essential medium (MEM) supplemented by Earles salts. The cells released into the medium were then sedimented by centrifugation and resuspended in MEM; cell number and viability were determined in a Burker chamber after conventional Trypan blue staining. The proportion of lymphoid cells was first assessed by counting in the chamber; the tumour cells were usually readily distinguishable from the lymphocytic cells by their larger size. The proportion of Trypan blue positive cells varied between 15 and 30 per cent. Most of the dead cells had the appearance of tumour cells. To verify further the frequency of lymphocytic cells, smear preparations were made from the cell suspensions, between 200 and 300 cells being scored after Giemsa staining. The tumour cells appeared larger than the lymphoid cells and had pale stained nuclei whereas the latter were darker. In the few cases of doubt as to whether a cell was lymphoid or not it was classified as a tumour cell.

Venous blood obtained from the patients about an hour before operation was defibrinated by gentle shaking in a beaker containing glass pearls. Three per cent gelatin dissolved in Hanks Tris buffer in a v/v ratio of 3 : 1 (blood: gelatin solution) was added and the erythrocytes were allowed to sediment at unit gravity for 45 minutes at 37° C; the supernatants were then collected, washed by centrifugation and resuspended in MEM. The number of lymphoid cells was then determined in a Burker chamber after crystal-violet staining. The frequency of contaminant non-lymphoid cells varied between 10 and 30 per cent. The viability of the cells, as determined after Trypan blue staining, exceeded 90 per cent.

Phytohaemagglutinin reactivity of cells in vitro. Culture conditions and measurements of isotope uptake by the cells has been described previously (EINHORN et coll. 1970). Various numbers of viable nucleated cells were suspended in 1.0 ml MEM containing 150 units of penicillin and 150 μg streptomycin/ml supplemented with 10 per cent serum from human donors of blood groups AB, Rh+ (the serum was previously heated to 56° C for 30 minutes and stored at —20° C before use). The cells were kept in 15 ml screw-cap glass tubes with

Table

Frequency of lymphoid cells in suspensions prepared from the tumours. The calculations were made after differential counts in a Burker chamber of living cells and on smear preparations with Giemsa staining. A tumour of the breast and an axillary metastasis were tested in Case 2.

Case	Percentage of lymphocytic cells	
	Smear preparation	Burker chamber
1	33	25
2 Tumour	29	55
Metastasis	78	74
3	23	20
4	30	24

a diameter of 15 mm and a rounded bottom. Each cell concentration was set up in quadruplicate and half of the cultures received phytohaemagglutinin (Bacto-Phytohaemagglutinin M, Difco Lab., Detroit, Mich, USA) at a final concentration of three per cent. After three days of culture at 37° C in a humified CO_2 atmosphere of five per cent each cell culture received 0.4 μCi ^{14}C-thymidine (50 mCi/mM, The radiochemical Centre, Amersham, England) in 0.1 ml MEM. The cultures were terminated twenty-four hours later and the cells washed in balanced salt solution by centrifugation before being precipitated in 5 % trichloroacetic acid. The precipitates were then dissolved in soluene, transferred to Packard scintillation vials containing scintillation fluid and the radioactivities measured in a Packard scintillation counter model 3380.

The $\log_{10}$ mean isotope incorporations of the duplicate cultures, expressed as counts per minute, were calculated. The values for the duplicate cultures that had received phytohaemagglutinin did not differ more than 0.2 $\log_{10}$ units, whereas cultures without this addition sometimes varied by a factor of up to 0.35 $\log_{10}$ units.

Results

Most of the lymphoid cells appeared small and non-blast transformed; occasional plasma cells were present in all tumour smears tested. The results of the differential counts are presented in the Table; some 20 to 80 per cent of the cells were lymphocytic and reasonable agreement was evident between the two techniques applied for differentiating the cells.

Lymphocytes obtained from the blood and tumour of the four different patients were tested on the same day and under identical conditions. The results

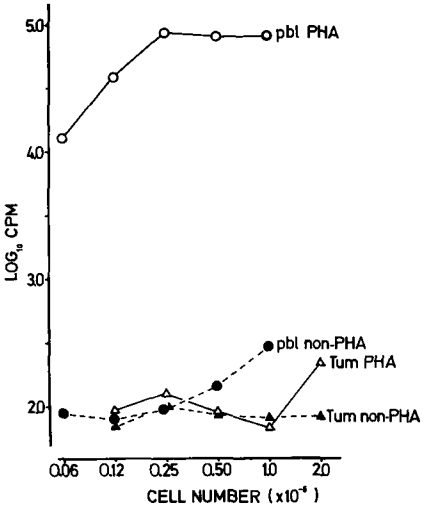

Fig. 1. Case 1. Incorporation of ^{14}C-thymidine in vitro expressed as $\log_{10}$ counts per minute by cells obtained from a breast carcinoma and autochthonous peripheral blood lymphocytes. Various numbers of cells were cultured in 1.0 ml medium in the presence or absence of phytohaemagglutinin; 25 to 33 per cent of the cells obtained from the tumour were of the lymphoid type. Pbl stands for peripheral blood lymphocytes and Tum for cells obtained from tumour.

obtained demonstrated that the lymphoid cells infiltrating the tumour exhibit an extremely low capacity to respond to phytohaemagglutinin as compared to autochthonous blood lymphocytes.

Case reports

Case 1. Female, aged 29. Radical mastectomy performed for a lump in the right breast detected a month previously and after delivery of a third child; the tumour had a diameter of about 8 cm with spread to the axillary lymph nodes. Histology: medullary type of carcinoma of the breast with extensive lymphocytic infiltration. The peripheral blood lymphocytes exhibited an increase in isotope uptake in response to phytohaemagglutinin by increasing the number of cultured cells from about 6×10^4 up to 2.5×10^5. A further increase in cell concentration failed to result in an increased ^{14}C-thymidine uptake. The cells obtained from the tumour exhibited only slight, if any, stimulation by phytohaemagglutinin. Some 30 per cent of the cultured cells from the tumour were of the lymphoid type (Table); hence 2×10^6 cultured cells from the tumour would contain lymphocytes in an amount equivalent to cultures with 0.5×10^6 blood lymphocytes, the difference in thymidine uptake by these two cell cultures being at least a hundredfold.

Case 2. Female, aged 44, with three weeks history of a lump in the right breast; this was removed by mastectomy. The mass measured about 4.5 cm in diameter; metastases were evident in the lymph nodes of the right axilla. Histology: medullary carcinoma with extensive lymphocytic infiltration. Blood lymphocytes, the presumed primary tumour and the cells from an axillary metastasis were examined for their responsiveness. Although both these tumours were heavily infiltrated with lymphoid cells (the metastasis containing more

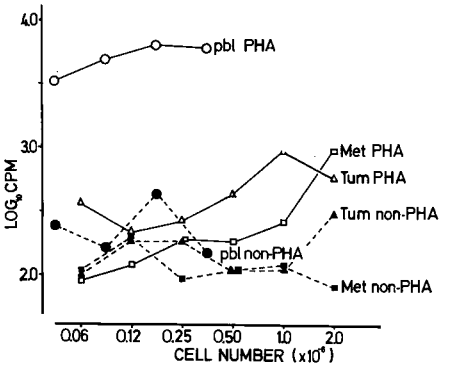

Fig. 2. Case 2. In addition to cells obtained from the tumour of the breast and the peripheral blood, an axillary metastasis was also tested; 29 to 55 per cent of the tumour cells and 74 to 78 per cent of the metastasis cells were of the lymphoid type. (Symbols as in Fig. 1.)

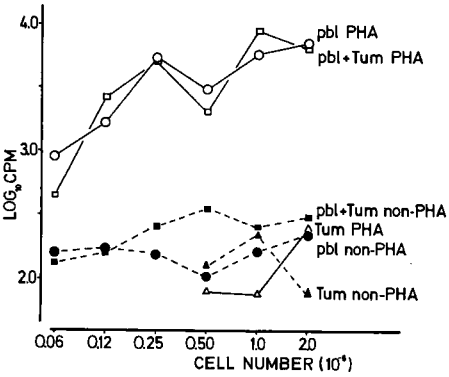

Fig. 3. Case 3. Cultures were also set up with varying numbers of peripheral blood lymphocytes admixed with a constant number of tumour cells (0.5×10^6); 20 to 23 per cent of the latter were of the lymphoid type. (Symbols as in Fig. 1.)

than 70 per cent lymphocytic cells) a higher thymidine incorporation in response to phytohaemagglutinin was obtained by the smallest number of cultured blood lymphocytes, i.e. 6×10^4, than the greatest number of cultured cells from both tumours or 2×10^6.

Case 3. Female, aged 47. Radical mastectomy for 2.5 cm lump in right breast, said to have been present for 2 weeks; no metastases. Histology: Poorly differentiated carcinoma, partly scirrhous, partly medullary and partly comedo. Extensive lymphocytic infiltration. The comparison of phytohaemagglutinin responsiveness between blood and tumour lymphocytes in Cases 1 and 2, indicated an extremely low reactivity of those obtained from the tumour. To test the possibility that this difference might have been due to the contaminating malignant cells, control cultures were included containing various numbers of blood lymphocytes admixed with a fixed number of cells from the tumour. The data obtained in this case indicated that the cells from the tumour failed to inhibit the phytohaemagglutinin responsiveness of the peripheral blood lymphocytes and confirmed the low responsiveness of lymphocytes from the tumour.

Case 4. Female, aged 51. Radical mastectomy following three weeks history of a lump measuring about 1.75 cm in diameter. No known metastases. Histology: Predominantly poorly differentiated mammary duct carcinoma with areas of comedo type and medullary carcinoma. Extensive lymphocytic infiltration. The cells from this tumour exhibited the highest phytohaemagglutinin response of those tested. However, comparison between the response of blood lymphocytes and an equivalent number of lymphocytes from the tumour confirmed the low reactivity of the latter cells. In agreement with the results from Case 3, the admixture of 0.5×10^6 cells from the tumour with the blood lymphocytes failed to decrease their responsiveness.

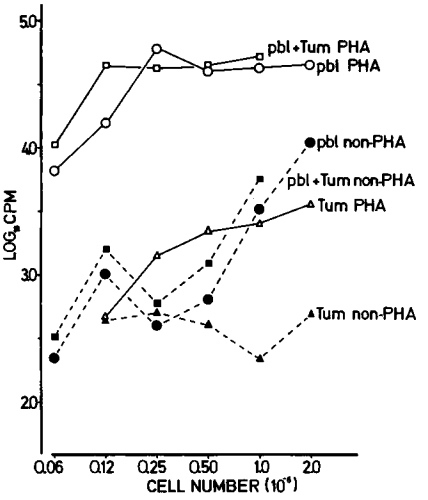

Fig. 4. Case 4. Same experimental method; 24 to 30 per cent of the tumour cells obtained were of the lymphoid type.

Discussion

The present investigation was aimed at the in vitro phytohaemagglutinin reactivity of lymphocytes infiltrating carcinoma of the breast in human subjects. Histology revealed that in two cases the tumours exhibited completely, and the other 2 cases partly, the morphology typical of medullary carcinoma. This condition is known often to exhibit extensive infiltration by lymphocytes and have a relatively favourable prognosis compared to other types of carcinoma of the breast (More & Foote 1949, Bloom 1970).

The lymphocytes infiltrating the tumour may represent cells in a stage of active sensitization against antigenically foreign structures on the malignant cells rendering them a specific cytotoxic potential. It appears that T-cells may be sensitized against alloantigens thereby achieving a cytotoxic potential against the cells bearing the antigens used for the purpose (Cerottini et coll. 1970, Blomgren et coll. 1970, Blomgren & Svedmyr 1971). The specific sensitizing step may occur in the absence of B-cells (Golstein et coll. 1972) and evidence also exists that the actual killing of the target cells by the sensitized lymphocytes may take place in the absence of B-cells (Golstein et coll. 1972). These observations suggest that it might be assumed that the lymphocytes invading neoplasms may be of thymic origin. Since phytohaemagglutinin is considered to have a high specific mitogeneic effect on T-cells (Rieke 1966, Greaces et coll. 1968, Blomgren & Svedmyr 1971) an attempt was made to compare the phytohaemagglutinin responsiveness of lymphocytes infiltrating a tumour and autochthonous blood lymphocytes in vitro. An extremely poor mitogeneic effect in the lympho-

cytes invading the former was evident in the 4 cases investigated. Blood lymphocytes from the same cases exhibited high thymidine incorporations in response to this mitogen. This may indicate that cells other than T-cells had almost selectively invaded the masses. Another explanation may be that they were of thymic origin but unresponsive to phytohaemagglutinin due to an active sensitization continuing against tumour associated antigens. The lymphocytes infiltrating the malignant tissue were, however, small in size with scanty cytoplasm and darkly staining nuclei; a high thymidine incorporation in vitro by the cells in the absence of phytohaemagglutinin, that might have been expected, was not observed. Another possibility may be that the malignant cells inhibit the phytohaemagglutinin response of the lymphocytes; this explanation seems less likely since the admixture of the cells to cultures containing blood lymphocytes failed to change the responsiveness.

It would appear that the majority of lymphoid cells infiltrating breast carcinoma are other than T-lymphocytes. In support of this view is the preliminary finding that such lymphocytes fail to bind sheep erythrocytes in vitro, which seems to be a specific characteristic of human T-lymphocytes (JONDAL et coll. 1972).

The results fail also to establish that most of the lymphocytes infiltrating the tumours are B-lymphocytes, although cells with a typical morphology of plasma cells were present in the growths. It is possible that these cells represent a cell population produced in the bone marrow (OSMOND & EVERETT 1964) not processed by the bursa equivalent. It is conceivable that T-lymphocytes exist in the malignant tissue and react against foreign antigens and thereby stimulating lymphocytes from bone marrow to invade the lesion. Such a massive migration of these lymphocytes has been observed in the delayed type of skin reactions known to be thymus dependent (LUBAROFF & WAKSMAN 1968).

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SUMMARY

Lymphocytes invading carcinoma of the human breast and autochthonous blood lymphocytes were compared regarding their stimulation in vitro by phytohaemagglutinin. Four cases are reported; in all these the infiltrating lymphocytes were poorly stimulated whereas the blood lymphocytes responded by a highly increased incorporation of isotope labelled thymidine. The results were interpreted as indicating that the tumours had almost selectively attracted the nonthymus dependent lymphocytes.

ZUSAMMENFASSUNG

Die Lymphozyten, die Krebsgewebe der menschlichen Brust infiltrieren, und die autochtonen Blutlymphozyten wurden hinsichtlich ihrer Stimulation in vitro durch Phythämagglutinin untersucht. Es wird über vier Fälle berichtet; in allen diesen Fällen waren die infiltrierenden Lymphozyten geringfügig stimuliert, während die Blutlymphozyten mit einem hochgradig gesteigerten Einbau von radioaktiv-gezeichnetem Thymidin reagierten. Die Resultate werden dahingehend interpretiert, dass die Tumoren nahezu selektiv die nicht-Thymus abhängigen Lymphozyten attrahiert hatten.

RÉSUMÉ

Les auteurs ont comparé l'effet de la stimulation in vitro par la phytohémagglutinine sur les lymphocytes envahissant le cancer du sein de la femme et sur les lymphocytes sanguins autochtones. Ils présentent 4 cas; dans tous ces cas les lymphocytes infiltrants étaient peu stimulés alors que les lymphocytes sanguins répondaient à la stimulation par une incorporation très augmentée de la thymidine marquée par un isotope. Ces résultats ont été interprétés comme indiquant que les tumeurs attiraient presque sélectivement les lymphocytes qui ne dépendent pas du thymus.

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