Localization of a Cathepsin-like and Aminopeptidase Activity in Various Solid Tumor Transplants

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SUMMARY

By use of chromogenic histochemical methods, a high activity of both leucine aminopeptidase and cathepsin C-like indoxyl esterase was demonstrated in stromal cells bordering the growing edge and marginating necrotic areas of various solid tumor transplants in mice and rats. The tumor tissue itself was found to be enzymatically inactive. The highest hydrolytic activity was localized in macrophages, mast cells, and fibroblasts. The enzymatic activity of stromal elements was considerably increased and expanded into tumors undergoing spontaneous regression, owing to proliferation of granulation tissue.

Studies on the histochemical distribution of proteolytic activity in solid tumor transplants by quantitative microdissection methods have firmly established the presence of high proteinase and peptidase activity in outer portions of actively growing neoplasms. The increased activity of arginase (14), dipeptidase, and "total" cathepsin (2, 22) in these areas has been attributed to the rapidly multiplying tumor cells of the "A" type (7) forming the invasive zone.

The exact localization of enzymatic activity at the cellular level seems possible only by the use of chromogenic reactions, the products of which can be microscopically traced within the intact tissue. The present study deals with the histochemical localization of leucine aminopeptidase and of a cathepsin-like activity in various tumor transplants in mice and rats and furnishes evidence of a high proteolytic activity present not in tumor cells but in stromal elements. Highly active stromal cells have been found to be intimately associated with the edge of actively growing tumors and also with granulation tissue invading transplants which are undergoing spontaneous regression.

MATERIALS AND METHODS

The various tumors studied are summarized in Table 1. Tumor tissue was aseptically transplanted into the right axillary subcutaneous tissue of recipient animals by trocar. The animals were fed "Nafag" pellets and had access to tap water. At the times indicated, the animals were killed by cervical dislocation, bled, and the tumors, together with surrounding noninvaded tissue, were excised. The removed tissue was cut into slices about 2 mm. thick, and alternating slices were quickly frozen in CO_2 or fixed in buffered formol-calcium at pH 7.0 for 16 hr. at 4° C.

Histochemical methods.—For the demonstration of leucine aminopeptidase (LAP) activity we used the azo dye-copper chelation method of Nachlas *et al.* (17). Fresh frozen 8-μ sections cut in a cryostat were incubated for 60 min. at 37° C. in the medium containing L-leucyl-β-naphthylamide (Borden Co., Philadelphia) as substrate. Cathepsin C-like indoxylesterase (IE) activity was demonstrated according to Hess and Pearse (13), with *o*-acetyl-4-chloro-5-bromoindoxyl used as a substrate. The reaction was performed on fixed frozen sections for 120 min. at 37° C. in the presence of 10^{-3} M cysteine, after preincubation of the sections with 10^{-5} M diethyl-*p*-nitrophenylphosphate (E600) for 60 min. at pH 7.4. Formalin-fixed slices were also paraffin-embedded, and sections, together with postfixed cryostat sections, were stained with the PAS-procedure, alcian blue, toluidine blue, and methyl green-pyronine.

RESULTS

The activity of both LAP and IE in the rapidly growing transplants (in contrast to the slowly

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growing Huggins fibroadenoma) was exclusively localized in stromal cells surrounding and irregularly invading the growing edge of the tumors (Fig. 1). In a given cell type, aminopeptidase and esterase activity were usually closely associated. The highest activity was found in macrophages, followed in decreasing order by mast cells (Fig. 3), fibroblasts, heterophilic granulocytes, and capillary endothelium. Apart from the fibroblasts, connective tissue fibers in the vicinity of the tumor margin gave a positive reaction for aminopeptidase. The intensity of the enzyme reaction as visualized by the methods used was dependent only on the number of inflammatory cells present enzymatic activity has been referred to by Nachlas *et al.* (17) and may be prevented by the use of a substrate (L-leucyl-4-methoxy-β-naphthylamide) capable of more rapid azo coupling (16). It is possible, however, that part of the diffuse staining pattern observed was the result of enzyme protein liberated from degenerating inflammatory cells.

Regressing tumors uniformly showed high activity of both LAP and IE in the granulation tissue. The activity was confined mainly to macrophages and fibroblasts present between tumor tissue remnants (Fig. 5). In both progressing and regressing transplants, a high hydrolytic activity

TABLE 1

HISTOCHEMICALLY EXAMINED SOLID TUMOR TRANSPLANTS

Tumor	No.	Host	Age of implant (days)	Growth
Crocker Sarcoma 180	2	Mouse (Swiss) ♂	10	Progr.
Adenocarcinoma E0771	4	Mouse (C57BL) ♀	6–12	Progr.
Ehrlich carcinoma (solid form)	4 1	Mouse (Swiss) ♂	13–16 13	Progr. Regr.
Walker carcinosarcoma 256	6 1	Rat (albino) ♂	6–15 11	Progr. Regr.
Uterusepithelioma T-8 Guérin	2 3	Rat (albino) ♂	12, 14 12–19	Progr. Regr.
Flexner-Jobling carcinoma	2	Rat (albino) ♂	19	Regr.
Cloudman melanoma S-91	2	Mouse (Swiss) ♂	11	Regr.
Harding-Passey melanoma	2	Mouse (Swiss) ♂	11	Progr.
Huggins fibroadenoma	1	Rat (Sprague-Dawley) ♀	63	Progr.

in a given area. Tumor cells were enzymatically inactive. This was particularly evident with the indoxyl method, which permits exact intracellular localization. In the outer portions of the tumor tissue, tumor cells could be easily distinguished from invading macrophages by the absence of an esterase reaction (Fig. 4). Hydrolytic activity, appearing within a narrow zone bordering enzymatically negative necrotic areas in central portions of several tumors, was due entirely to inflammatory cells (Fig. 2). A slight staining by azo dye of the outer tumor zone in the vicinity of strong stromal activity, as demonstrated by the aminopeptidase method, might be due to adsorption of enzymatically liberated naphthylamine before the completion of azo coupling. This diffusion artifact appearing at the margin of high of stromal cells was paralleled in fixed sections by a high mucopolysaccharide content of connective tissue ground substance as revealed by strongly positive PAS, alcian blue, and metachromatic staining. This finding is in keeping with observations made by Catchpole (8), who described increased amounts of water-soluble glycoproteins in the connective tissue bordering and abutting on transplanted tumors.

A discrepancy between the localization of the two enzyme reactions used was found in the slowly growing, nonmalignant Huggins fibroadenoma. The fibrous part of this tumor showed an extremely high LAP activity (Fig. 6), whereas IE activity was limited to scattered macrophages. The glandular structures exhibited strong activity of both LAP and IE.

DISCUSSION

A high activity of both aminopeptidase and a cathepsin-like esterase has been demonstrated by histochemical methods in various stromal cells of the tissue surrounding the growing edge and limiting necrotic areas of actively growing epithelial tumor transplants. A similarly high activity was displayed by elements of the granulation tissue invading regressing transplants. These findings are corroborated by the results of quantitative histochemical-biochemical methods (2, 22) as far as broad localization is concerned, but they do not support the view that outgrowing tumor cells are mainly responsible for the high protease and peptidase activities present in the invasive marginal tumor zone. This difference in interpretation depends mainly on two factors: first, it seems unlikely that tumor cells can effectively be separated from inflammatory cells by microdissection, since mesenchymal elements occur mingled with the neoplastic cells. Second, there are marked differences between the methods and the substrates used for enzyme assay by biochemical methods and by chromogenic histochemical technics, respectively. The two types of methods have in common a relatively low substrate specificity. In contrast to the *in vitro* determination of "overall" catheptic activity by measurement of split products of substrates like casein, nitrocasein, edestin, hemoglobin, the visualization of cathepsin C-like activity by the indoxyl method depends on the nonspecific esterase activity of many proteolytic enzymes (18). In the case of intracellular proteases, this esterase activity is partly resistant to organo-phosphorus inhibitors (13). L-leucyl-β-naphthylamide apparently is specifically hydrolyzed by leucin aminopeptidase (17). In the purified state, this enzyme is free of esterase activity (21) but does not represent a biochemical entity (24).

Leukocytes have been shown to contain various peptidases (9). Histochemically, a high activity of hydrolytic enzymes (esterases and phosphatases) has been demonstrated in macrophages, histiocytes (12, 13, 25), blood monocytes (5), and in granulocytes (23). Mast cells exhibit LAP activity (4) and contain an enzyme similar to chymotrypsin (1). Preliminary experiments have demonstrated a high activity of both LAP and IE in macrophages, fibroblasts, and, to a lesser degree, in granulocytes forming part of aseptic subcutaneous granulomas in the rat. These findings are in keeping with recent observations by Monis *et al.* (16) on high LAP activity in cutaneous granulation tissue and in tissue culture of fibroblasts.

The conclusion drawn from the present findings on solid tumor transplants cannot be applied directly to other kinds of experimentally induced or spontaneous tumors. Catheptic activity has been reported to be higher in spontaneous neoplasms than in tumor transplants (15). Ehrlich ascites tumor cells contain very active labile peptidases capable of cleaving alanylglycine and glycylleucine, but contain comparatively little stable enzyme hydrolyzing leucineamide (19). In various human carcinomas a histochemical reaction for LAP can be demonstrated in epithelial portions as well as in the stromal tissue (3, 6, 11, 16, 26). The epithelium of human carcinoid tumors possesses highly active cathepsin-like IE (20). A close correlation does exist between the proteolytic activity visualized histochemically and the high activity of several proteases demonstrated by biochemical methods in regressing tumor transplants, namely, the Flexner-Jobling carcinoma (10) and the Walker carcinosarcoma 256 (2).

The present histochemical observations indicate that enzymatic activity of stromal tissue is not related to invasive properties of transplanted tumors but is dependent on the extent of nonspecific inflammatory response and on connective tissue proliferation. This conclusion is supported by recent studies on stromal LAP activity of human neoplasms (16).

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FIG. 1.—Solid Ehrlich carcinoma. High leucine aminopeptidase activity in stromal cells bordering and partially infiltrating the growing edge (lower third of fig.). Mag. ×200.

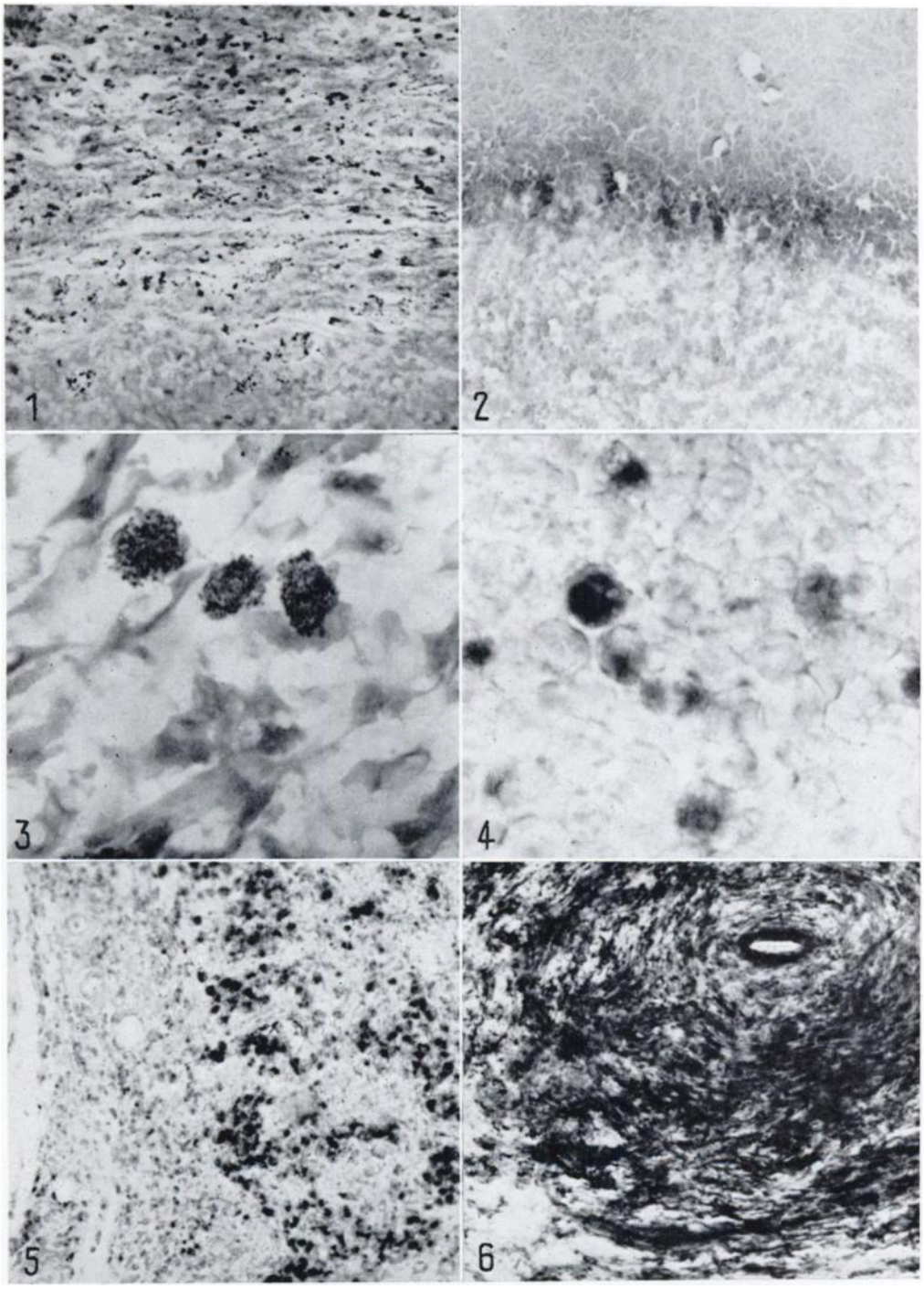
FIG. 2.—Walker carcinosarcoma 256. Leucine aminopeptidase activity in inflammatory zone bordering necrotic tumor tissue. Mag. ×140.

FIG. 3.—Same tumor as Figure 2. High activity of leucine aminopeptidase in mast cells of marginal stromal tissue. Comparatively weak activity in fibroblasts. Mag. ×600.

FIG. 4.—Cathepsin-like esterase in macrophages invading the outer zone of a mouse adenocarcinoma E0771. Indoxyl-method. Mag. ×560.

FIG. 5.—Regressing Flexner-Jobling carcinoma. Indoxylesterase activity in macrophages and fibroblasts forming part of the granulation tissue which is invading the tumor remnant. Mag. ×140.

FIG. 6.—Huggins fibroadenoma. Highly active leucine aminopeptidase in fibrous and glandular portions. Mag. ×85.



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