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# Leucine aminopeptidase : its role in diseases processes

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# LEUCINE AMINOPEPTIDASE,

# ITS ROLE IN DISEASE PROCESSES

By

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Leucine aminopeptidase (LAP) is a protolytic enzyme which has been popularized the past several years for its possible role as a diagnostic aid in the discovery of early carcinoma of the pancreas. However, elevated serum levels of this enzyme have also been noted in normal pregnancies.[1-2] Several investigators[3] have attempted to correlate this enzyme's activity with the invasive properties of malignant tumors. On the other hand, the findings of Monis, Nachlas, Seligman[4] and of Hess,[5] seem to demonstrate clearly that the stromal LAP activity is part of the host's response to the invasive tumor cells. Thus, there are many conflicting articles in today's literature concerning the role of LAP in disease. Such diversity of characteristics and controversy of facts brings about the necessity for a review of the literature concerning the activity of this enzyme in serum and in tissue, and an effort made, utilizing the experimental laboratory, to demonstrate its role in disease processes.

Early work on leucine aminopeptidase began in the mid 1940's by Zamecnek,[6] et. al., who reported an increase, of 6-20 fold, of an aminopeptidase in lymph and sera of dogs which had been experimentally burned. Interestingly enough, it was noted that the white cell count in the collected lymph rose from 2-4 fold. The substrate used was l-leucylglycylglycine.

Since that time, LAP has been studied in serum, urine, cerebrospinal fluid and in tissue. The early works of Goldbarg and Rutenburg,[7] pioneers in this field, especially relate to LAP activity in serum and urine. The method used for serum assay is similar regardless of

the investigator. The enzyme, being present in the serum, is added to a substrate and the product of this reaction chelated with copper to give a colorimatic end point.

Hammond,[8] et. al., using the methods of Goldbarg and Rutenburg noted much spontaneous variation from day to day in their control subjects. They concluded that serum LAP tests lacked sensitivity and specificity, and did not seem to relate to biliary obstruction.

Shay, Sun, and Siplet[9] also using the method of Goldbarg and Rutenburg concluded that neither the pancreas, nor carcinoma of the pancreas, could be responsible for elevations of serum LAP, but rather such elevations are the product of some type of cholestasis.

Pineda,[10] et. al., using the Goldbarg-Rutenburg method, concluded that patients with carcinoma of the head of the pancreas or extrahepatic biliary tract had extremely high serum LAP levels with and without jaundice. Such serum levels were also elevated with carcinoma of the tail of the pancreas. He observed moderate elevations in patients with acute or drug induced hepatitis, and he felt they could differentiate carcinoma of the head of the pancreas from acute viral hepatitis on this basis. They concluded that a high serum LAP, that is, greater than 500 G-R units, is indicative of carcinoma of the pancreas.

Miller,[11] et. al., using the Goldbarg-Rutenburg method, concluded serum LAP was of no value as a diagnostic aid in carcinoma of the pancreas.

Banks,[12] et. al., also using the Goldbarg-Rutenburg method, concluded that persistent elevations (greater than 450 G-R units)

of serum LAP for greater than one week strongly suggested a carcinoma of the pancreas rather than obstruction by common duct stone.

Hoffman,[13] et. al., using an l-leucylglycine substrate rather than the more conventional l-leucylnaphtylamid concluded that the degree of serum LAP elevation could not be used to differentiate obstructive jaundice from hepatocellular disease.

Regardless of methodology, it at first seemed, as noted above, that there was a correlative rise in serum LAP in diseases of the hepatobiliary tract, especially carcinoma of the pancreas. Despite enthusiasm for a laboratory test to diagnose early carcinoma of the pancreas, subsequent investigators have failed to substantiate this point. It is, however, fairly well agreed that serum LAP elevates significantly in diseases of the hepatobiliary tract (including carcinoma of the pancreas) and that the probable source of such enzyme release is in the biliary radicals. Nonetheless, diseases of the hepatobiliary tract cannot be satisfactorily separated by the serum LAP elevations they produce as similar elevations are seen in patients with infectious hepatitis, drug induced hepatitis, intra-hepatic ductal obstruction, common duct obstruction or carcinoma of the pancreas.

This picture is further complicated by the fact that serum LAP elevates significantly during normal pregnancy. Serum LAP returns to normal within the eighth day post partum and is normal in newborn infants. Current investigators feel the source of this enzyme elevation is the placenta.

Turning, then, to tissue LAP, in 1956 Burstone,[14] et. al.,

demonstrated proteolytic activity in human neoplasma by histochemical means. In the next few years Burstone, as well as others,[15-20] worked specifically with LAP in an attempt to localize its position within the cell and explain its presence.

Tissue LAP activity has been studied fairly extensively in inflammatory processes. In three-day-old experimental cutaneous wounds in rats, Monis,[4] et. al., found connective tissue cells (granulation tissue) to be most active. In the seven-day-old wound, the granulation tissue increased in cellularity with corresponding increase in tissue LAP activity. Fibroblasts, macrophages and neutrophils stained intensely. They concluded that an increase in LAP activity in connective tissue was probably the normal physiologic mechanism for the proliferation of connective tissue. Increased serum LAP levels correlated with the increased tissue LAP activity.

Bajusz and Jasmin[21] performed an interesting experiment by ligating the left circumflex coronary artery in rats and staining the myocardium at various intervals for different proteolytic enzymes. They found that tissue LAP activity reached a peak at the site of infarction at 72 hours. The line of demarcation between dead and viable tissue was quite discrete. The neutrophils which infiltrated the infarcted area stained intensely for tissue LAP. This led them to suggest the possibility that the proteolytic activity of LAP was necessary to promote the lysis of non-viable myocardial cells as a part of the normal healing process.

Rosenholtz and Wattenberg[22] have demonstrated gallbladder carcinoma

tumor cells stained intensly for LAP. They have also shown such activity in gastric carcinoma tumor cells. With carcinoma of the breast only moderate activity (in one specimen) was observed; and in five metastatic breast carcinoma lesions (to the liver) only one showed positive LAP activity. In carcinoma of the pancreas, only one specimen showed positive activity.

In another study by Wattenberg,[23] an attempt was made to corelate intestinal metaplasia with carcinoma of the stomach utilizing LAP as the correlating factor. He was able to demonstrate high LAP activity in the epithelium of the mucosa of the small intestine, in intestinal metaplasia of the stomach, and in tumor cells of approximately one-third of the gastric carcinomas studied. No LAP activity was observed in the epithelium of normal gastric mucosa. Thus, lending proof that intestinal metaplasia is a forerunner of gastric carcinoma. More important, perhaps, is that LAP has been positively stained within tumor cells, some of which contain little or no LAP activity in the normal state. Might this enzyme then, be related to the invasiveness of tumors?

Conventional histologic investigation has repeatedly shown morphologic alterations in the stroma at the border between neoplastic cells and the host's tissue. The question remains whether to ascribe these changes to the host's reaction to the invasion by neoplastic cells or to the act of invasion by the tumor itself.

With the development of histochemical methods for localizing aminopeptidase activity, the techniques were soon applied to neoplastic

tissue. A zone of increased enzyme activity was observed in the stroma at the periphery of many, but not all, malignant neoplasms. One group[4] reported increased LAP activity in the connective tissue of benign neoplasms (colloid hyperplasia of the thyroid, benign prostatic hypertrophy) as well as inflammatory diseases (peptic ulcer, ulcerative enteritis). It was concluded that in view of the enzyme abundance in proliferating connective tissue of neoplastic, inflammatory and repairative processes, there was no reason to associate increased stromal LAP activity, of malignant processes, with the invasive properties of a tumor.

However, other workers[3] observed the sites of LAP activity in over 120 surgical specimens of human benign and malignant tumors. They noted a variability in enzyme activity from 0-4+ and interpreted this variability to indicate the possibility of LAP being one of a series of proteolytic enzymes involved in invasiveness. They were unable to demonstrate elevated serum activity in inflammatory lesions and, therefore, concluded that the enzyme was specifically related to malignant neoplastic cell invasion.

Thus, two conflicting views concerning the role of LAP in the invasiveness of malignant tumors have evolved: (a) The enzyme is part of the host's general reaction to injury, and (b) the enzyme is part of the tumor's invasive mechanism.

The best work done to date to resolve this conflict has been done by N. K. Mottet.[24] His experiment utilized a human epidermoid carcinoma (H.Ep. #3) selected for its vigorous invasive properties and its morphologic and immunologic stability in spite of several years of serial

transplantation. The experiment consisted of four parts:

Group I. H.Ep. #3 on Chorio-allantoic Membrane of Chicken Embryos.

Group II. H.Ep. #3 in Conditioned Weanling Wistar Rats.

Group III. H.Ep. #3 in Conditioned Nonweanling Wistar Rats.

Group IV. H.Ep. #3 in Nonconditioned Weanling Wistar Rats.

Conditioning in the weanling rats was accomplished by 150r of total body x-radiation. On the third post-irradiation day 0.1 cc. of H.Ep. #3 was injected in each flank. Three milligrams of cortisome was injected subcutaneously at the time of transplantation and every second day thereafter for four doses.

Nonweanling rats were conditioned in the same manner. These were included in the experiment as it has been reported that rats beyond the weanling stage were not as susceptible to conditioning as weanlings.

In Group I (CAM) there was extensive infiltration with no elevated stromal LAP activity adjacent to the tumor.

Group II (Conditioned Weanling Rats) showed extensive infiltration and metastasis with no zone of increased LAP activity.

In Group III (Conditioned Nonweanling Rats) rats sacrificed at 17 days had tumors which were grossly cystic with central necrosis and a dense peripheral fibrous capsule. This fibrous capsule was intensely reactive for LAP activity.

Group IV (Nonconditioned Weanling Rats) sacrificed after seven days revealed tumors with a necrotic core and a surrounding fibroproliferative zone. This zone demonstrated elevated LAP activity similar to Group III.

The conclusion drawn is that LAP activity represents the host's reaction to the invading neoplasm and not part of the mechanism by which malignant neoplasms invade host tissue.

In summary then, we can reasonably assume the following facts to be true:

1) Serum LAP elevates in diseases of the hepatobiliary tract whether it be intra- or extra-hepatic biliary obstruction or intrinsic liver disease, e.g. viral or drug induced hepatitis.
2) Serum LAP is elevated in normal pregnancies returning to baseline values within eight days post partum. The elevation generally is believed to be placental in origin.
3) Tissue LAP is found in many tissues, principally kidney tubular cells, small bowel mucosa, pancreatic acinar cells, biliary ductal cells and the epidermis.
4) In many sections of inflammatory or reparative tissue, macrophages, fibroblasts, and neutrophils stain intensely.
5) Tissue LAP is present within some tumor cells and in the stroma around neoplastic foci. In the periphery it functions as the host's general response to injury rather than as a function of the tumor's invasiveness.

Even is the above assumptions are true, questions remain concerning the function and purpose of this enzyme in a biologic system. For example, what is the source of LAP in an inflammatory or reparative process? How does it come to be concentrated in an area of injury? Certainly the work of Bajusz and Jasmin[21] indicate it is carried to the

site of injury by neutrophils. If this then were true, one should be able to stain neutrophils in the peripheral blood for LAP and perhaps as well in bone marrow.

To carry out this hypothesis, it becomes necessary to first review the knowledge accrued to date concerning the techniques of staining LAP, perfect them in the laboratory and then apply them to peripheral blood and bone marrow.

Summarizing recent literature, one becomes readily cognizant of the following facts concerning the staining of tissue LAP:

1) The tissue must be relatively fresh (within 4-6 hours).
2) It must be quick frozen (minus 80 degrees C) without using any preservatives.
3) Sections are cut in the cryostat.
4) A variety of substrates are satisfactory, l-leucyl-2-naphthylamide being a favorite.
5) Acetate or phosphate buffers are used with pH optimum of 6.0 for the former and 6.5 for the latter.
6) 0.2 M sodium chloride is added to decrease the solubility of LAP.
7) Potassium cyanide is added to 'stimulate" the activity of LAP.
8) A diazo dye is utilized, for example, diorthoanisidine.
9) After a diazo dye-naphthylomide bond is formed, the molecule is chelated with copper for stability.

Let us now consider these factors in more detail. First of all, there is not much question that LAP activity drops off fairly rapidly post mortem; that is, appreciable loss of activity is noted

in 12 hours. This decrease in activity correlates with the rate of autolysis.

Tissue LAP, or at least a portion of it (lyo) is very soluble, especially in solutions commonly used as fixative, e.g. formalin, acetone and methanol. This solubility interferes greatly with tissue localization as the lyo form diffuses out of the cell resulting in a homogenously stained section without morphological localization. This problem has been solved in part by the addition of free ions to the incubating solution, namely sodium chloride (0.85%) to reduce solubility, and by using fresh-frozen cryostat tissue sections. Tissue must be quick frozen to a temperature of minus 80 degrees C. then it may be allowed to slowly thaw to minus 20 degrees C. and then may be preserved for several months.

As mentioned above, the first substrate to demonstrate LAP activity was l-leucylglycylglycine. Since then many others have been tried but l-leucyl-2-naphthylamide has been the most popular. Recently, however, Nachlas,[15] et. al., has shown that l-leucyl-4-methoxy-2-naphthylamide will couple 40 times faster with the diazo dyes giving much better tissue localization.

Acetate and phosphate buffers are used fully interchangeably. The important factor here being pH. As a general rule, enzymes of this nature work best in an alkaline pH range (7.0-8.5). However, diazo coupling (the in situ reaction) proceeds best in a slightly acid pH range. The acetate optimum being 6.0, the phosphate 6.5.

Potassium cyanide is added to the incubating solution to "stimulate"

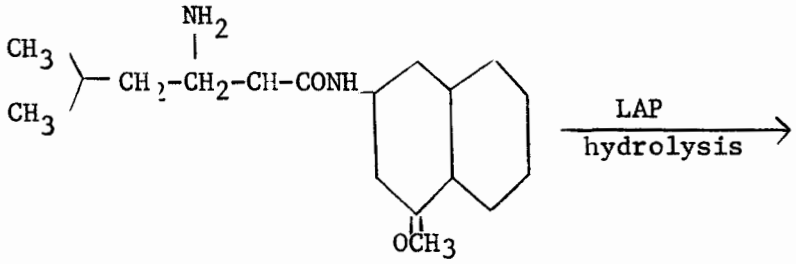
the activity of LAP. The mechanism here is unknown. More is known, however, about the role of metals in this enzyme's activity. LAP is a metallo-enzyme; that is, it requires a metal to be present within its structure before it becomes active. The metal required is magnesium or manganese or cobalt. Of these, magnesium has been studied most and has been the least rewarding. On the other hand, cobalt has been studied least and is the most interesting. Attempts at adding magnesium to incubating solutions were all unsuccessful in producing a more satisfactory stain. It has been shown by Walker,[25] et. al., that natural tissue concentrations of magnesium are adequate to activate this enzyme.

Turning then to cobalt, Decker and Dicken,[18] had postulated the presence of two enzymes, namely LAP-I and LAP-II, based in part on the activation of LAP-II by cobalt. LAP-II is heat stabile; its pH optimum is 7.2; it is activated by cobalt and it is not affected by cyanide. LAP-I is heat labile, pH optimum is 6.5 to 7.2; it is activated by magnesium or manganese and it is stimulated by cyanide.

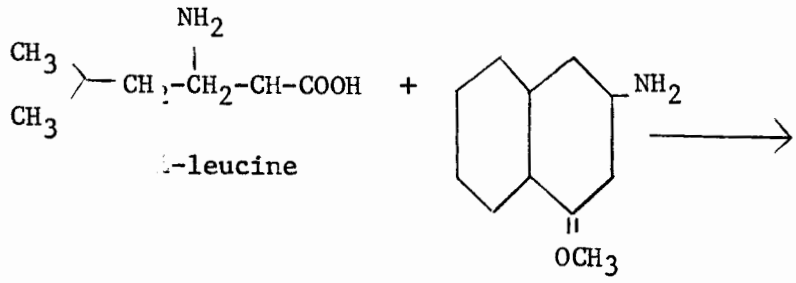
Gleanner,[3] et. al., on the other hand, reports no activation of LAP using magnesium, manganese or cobalt.

Several diazo dyes have been used in LAP techniques. Of these, Fast Blue B and Garnet GBC are the most popular. The reaction, which is similar in tissue and in serum, proceeds as follows. LAP is present in the tissue and is activated by magnesium or cobalt. When in contact with the substrate it hydrolyzes the l-leucine - naphthylamide bond.

The freed naphthylamide then couples with the diazo dye resulting in a color complex. This complex, however, is unstable until chelated with cupric sulfate. The reaction is diagrammed below using l-leucyl-4-methoxy-2-naphthylamide as a substrate.

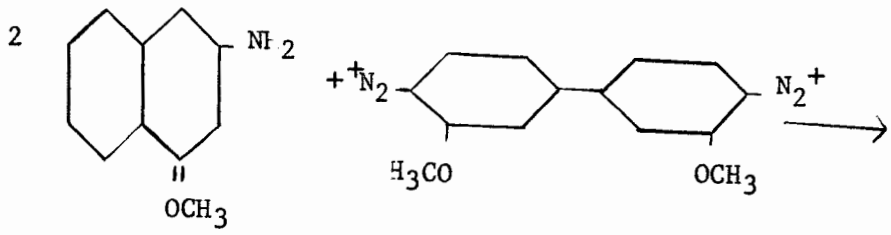



l-leucyl-4-methoxy-2-naphthylamide

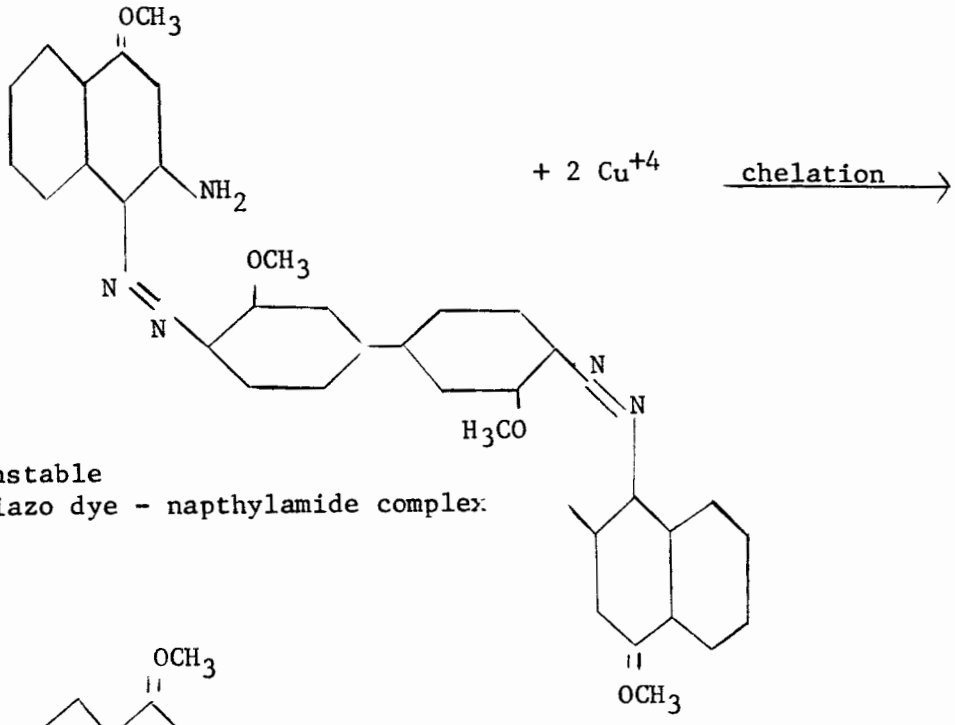



l-leucine

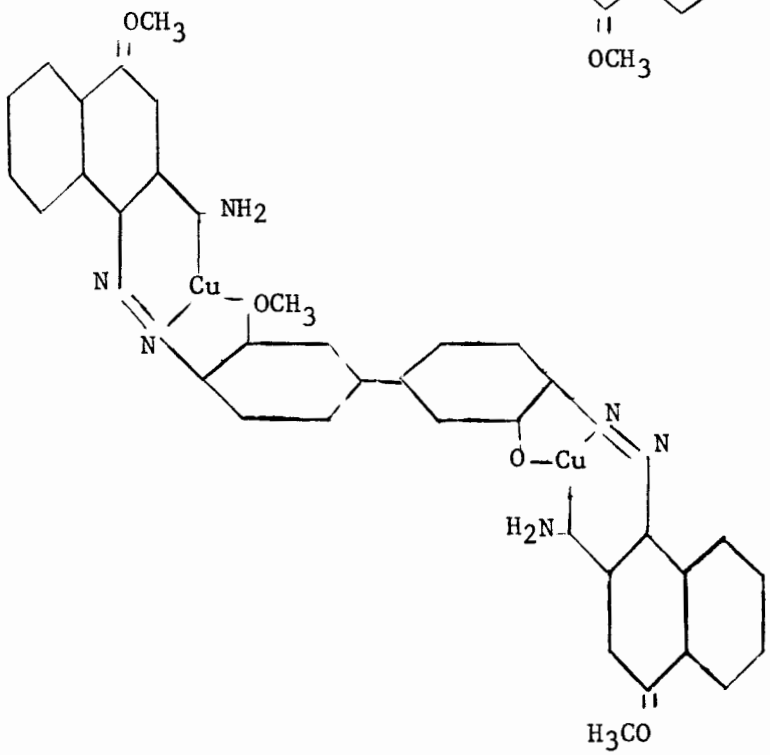
4-methoxy-napthylamide




tetrazoatized diorthoanisidine




Unstable
Diazo dye - napthylamide complex




Stable Diazo dye - copper - napthylamide complex

The attempt to stain white blood cells, especially neutrophils, for LAP is based on the assumption that tissue LAP is part of the host's general reaction to injury. Insoluble LAP may be transferred to areas of injury via neutrophils to aid in the healing process. To be sure, as mentioned earlier, in all tissue preparations neutrophils have always stained intensely. However, it is not known whether they pick this enzyme up at the tissue site or whether they carry it to the tissue site in the blood stream. The experimental methodology is described below.

The tissue chosen for control studies was human kidney because of its documented richness for LAP. Kidney was taken from postmortem examinations within 2-3 hours after death or taken in the operating room within the hour. The specimens were then blocked and frozen with carbon dioxide to minus 80 degrees C. then allowed to warm gradually to minus 20 degrees C. in the cryostat. Sections were then cut for use in the cryostat. Slides were placed in one of several incubating solutions as will be shown below. After incubating at 37 degrees C. for 5-45 minutes, the slides were rinsed in saline for 2 minutes and then transferred to a 0.1 M cupric sulfate solution for 3 minutes. The slides were then dehydrated in graded alcohol solutions, cleared in xylene and mounted. With each set of slides parallel, hematoxylin and eosin stained sections served as controls.

Incubating Solution No. 1:

1) L-leucyl-4-methoxy-2-naphthylamide hydrochloride. 4 mg./ml. 2.0 ml.
2) Acetate buffer (0.1 M) pH 6.5. 10.0 ml.

3) Sodium chloride (0.85%). 8.0 ml.

4) Potassium cyanide (2 x 10 -2 M). 1.0 ml.

5) Tetrazoatized diorthoanisidine. 20.0 ml.

Incubating Solution No. 2:

The same as No. 1 with phosphate buffer at pH 6.5 in place of acetate.

Incubating Solution No. 3:

The same as No. 1 with acetate buffer at pH 6 rather than pH 6.5.

Incubating Solution No. 4:

The same as No. 3 with cobalt chloride (4 x 10 -3 M) added.

Incubating Solution No. 5:

1) L-leucyl-4-methoxy-2-napthylamide hydrochloride 50 mg.

2) Acetate buffer (0.1 M) pH 6.0. 50 ml.

3) Tetrazoatized diorthoanisidine 50 mg.

Peripheral blood smears were incubated in the same solutions as the kidney sections. Solution No. 5 was especially adapted for peripheral blood staining from work done by Suzuki[26] on an azo dye method for staining leukocyte alkaline phosphatase. Freshly prepared peripheral blood smears are allowed to dry in air for 10 minutes, then fixed in neutral formalin vapor for 10 minutes, and then allowed to dry in air for one hour. The slides are then placed in the incubating solution for from 10-90 minutes. The slides are then rinsed in saline for 2 minutes, chelated with cupric sulfate for 3 minutes and then counter-stained in Meyer's hematoxylin for 30 minutes.

The results are summarized in the following chart.

## RESULTS

| | SOLUTIONS | TISSUE | INCUB. TIME | MICROSCOPIC PRESERVATION OF MORPHOLOGY | MICROSCOPIC LOCALIZATION |
|---|---|---|---|---|---|
| 1. | #1 | Kid. | 5 min. | fair | fair to poor |
| 2. | #1 | Kid. | 10 min. | fair | fair |
| 3. | #1 | Kid. | 15 min. | poor | poor |
| 4. | #1 | Kid. | 45 min. | poor | poor |
| 5. | #2 | Kid. | 5 min. | fair | fair |
| 6. | #2 | Kid. | 10 min. | good | fair |
| 7. | #2 | Kid. | 15 min. | poor | poor |
| 8. | #2 | Kid. | 45 min. | poor | poor |
| 9. | #3 | Kid. | 10 min. | good | good |
| 10. | #4 | Kid. | 10 min. | good | good |
| 11. | #1 | P.B. | 10 min. | good | absent |
| 12. | #2 | P.B. | 10 min. | good | absent |
| 13. | #3 | P.B. | 10 min. | good | absent |
| 14. | #3 | P.B. | 90 min. | good | absent |
| 15. | #4 | P.B. | 10 min. | good | absent |
| 16. | #5 | P.B. | 90 min. | good | poor |

As can be readily noted from the chart, solutions #3 and #4 gave the best results on the kidney sections. Neither appeared superior to the other. (Solution #4 contained CoCl) No completely satisfactory results were obtained with the peripheral blood smears. However, it is interesting to note that many neutrophils appear to have a pink cytoplasm while the majority have a clear cytoplasm. Thus, one wonders if those cells with pink cytoplasm are due to staining artifact or to soluble LAP diffusely present in the cytoplasms. Certainly in the control slides stained only with Meyer's hematoxylin no pink cytoplasm was observed. In no instance were definite granules seen in neutrophilic cytoplasm.

Normal blood (WBC less than 10,000) was used for the majority of smears. However, slides were also stained with blood showing leukocytosis, left shift and toxic granulations. Histochemically, the latter were no more reactive than the former.

## SUMMARY AND CONCLUSIONS

The purpose of this paper has been to review the literature concerning the role of LAP in disease processes and to lend some new information concerning the mechanism by which this role is played.

Early enthusiasm for the serum assay of LAP being a diagnostic tool in early diagnosis of pancreatic carcinoma was ill founded as the majority of subsequent investigators have refuted its value.

There is strong evidence to support the concept that tissue LAP acts as a part of the host general response to injury. However, the

mechanism of this response remains unclear. To be sure the results of the experimental work contained herein are not conclusive. That is, one cannot definitely state that LAP is carried in peripheral blood cells. However, one can be reasonably assured that LAP is present in normal serum and that it is present in neutrophils found in tissue.

It is reasonable, then, to assume its presence in neutrophils of peripheral blood. Certainly if this assumption were true, it would lend considerable support to the argument that LAP serves in the body's general response to injury. Furthermore, one may say it appears the mechanism of the LAP response is mediated via the peripheral neutrophils.

PHOTO KEY

Fig. 1. Kidney tubules (H & E control) - Normal tubular structure. (100x)

Fig. 2. Kidney glomerulus (LAP stain). Note the scant activity within the glomerulus. (400x)

Fig. 3. Kidney tubules (LAP stain). These tubules are intensely stained (red granules) for LAP and show good localization. (400x)

Fig. 4. Peripheral blood (Meyer's hematoxylin control). This cell was stained only with the counterstain. (1000x)

Fig. 5. Peripheral blood (LAP stain). The lymphocyte (center) shows no reaction. The adjacent neutrophils have a pink cytoplasm (compared with the control above).

Fig. 6. Peripheral blood (LAP stain). The cytoplasm of these cells is fairly clear; however, there is a suggestion of a pink granule at the periphery of the nucleus of the lower neutrophil. (1000x)

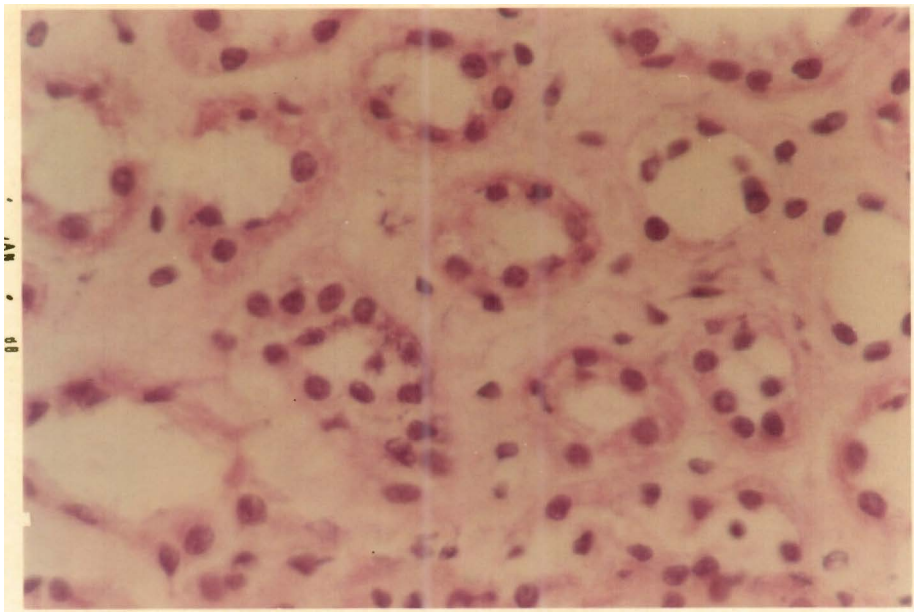


Fig. 1.

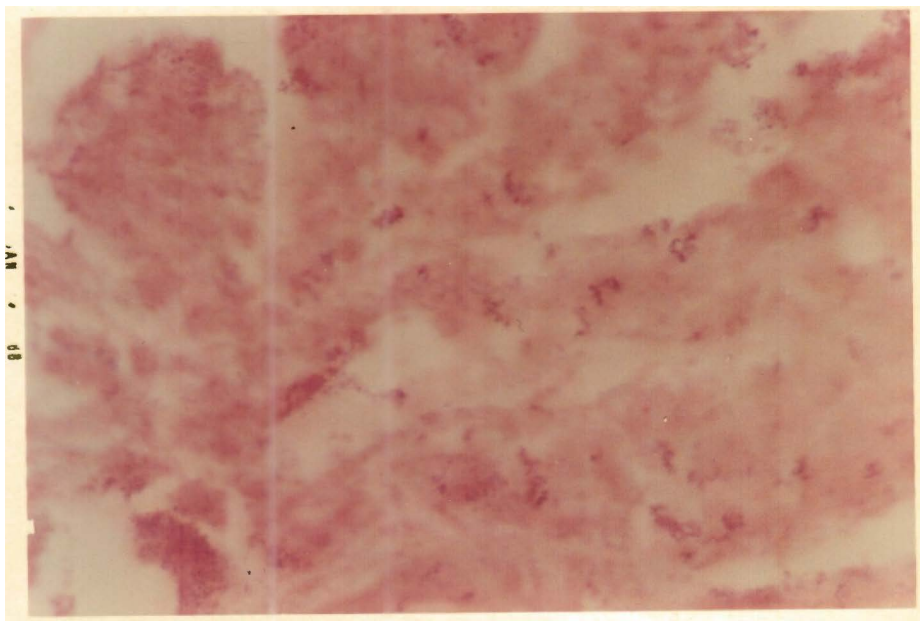


Fig. 2.

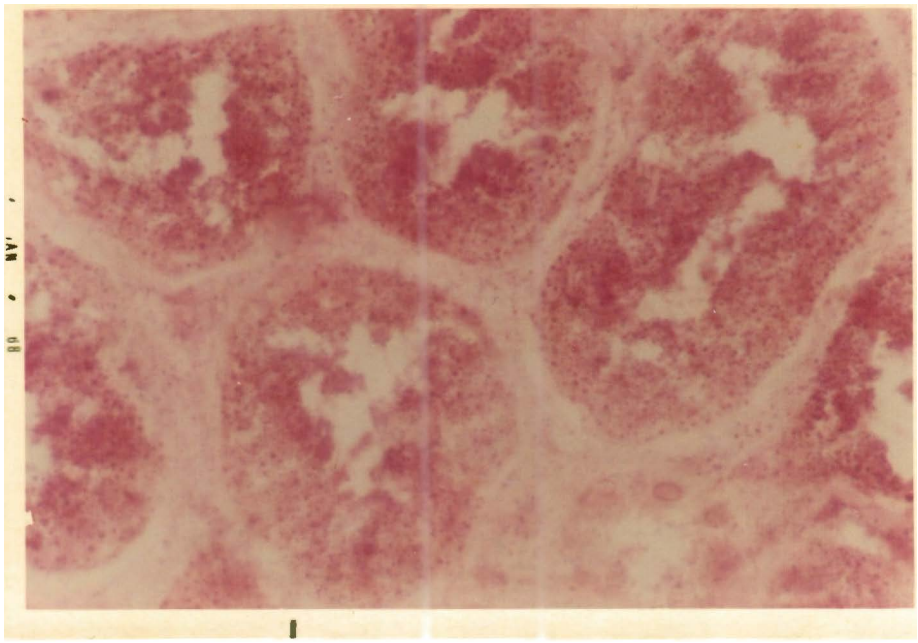


Fig. 3.

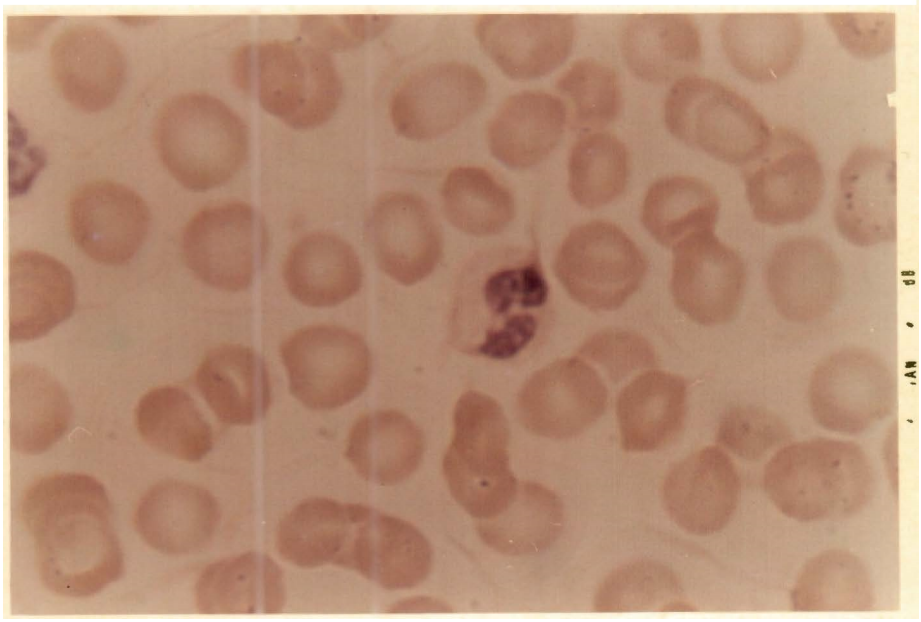


Fig. 4.

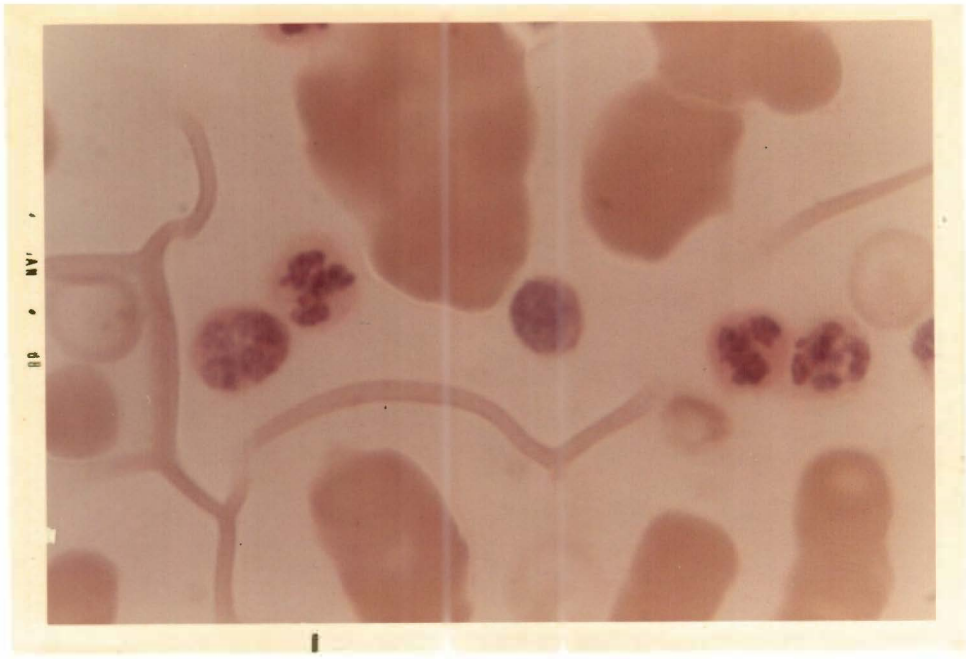


Fig. 5.

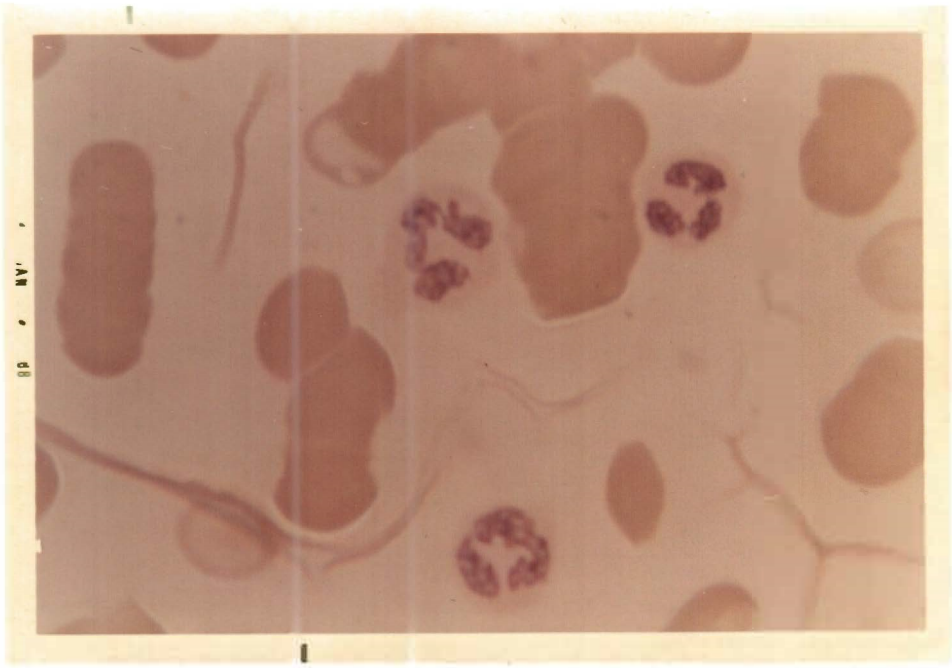


Fig. 6.

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