HISTOCHEMICAL DISTRIBUTION OF GUANASE IN HUMAN TISSUES WITH GUANINE IN BICINE BUFFER AS SUBSTRATE


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Histochemical studies of human guanase have seldom been undertaken, in part because of technical difficulties which result in heavy background staining. We previously reported a modified procedure in which the methodological inadequacies had been overcome. In this report, the modified technique has been applied to determine the distribution of guanase in normal human tissues and in cases of chronic hepatitis, hepatocellular carcinoma and metastatic tumors in liver (adenocarcinoma of colon). Guanase was present within the cytoplasm of hepatocytes throughout the entire lobule. Portal components (bile ducts and veins), fibrous tissue and inflammatory cells were non-reactive, and the enzyme was absent from hepatocyte nuclei in the normal liver. However, in the case of non A non B hepatitis nuclei presenting guanase activity or otherwise were identified. Guanase activity was observed for primary liver tumor but not for metastatic tumor. Moreover, guanase activity was observed in proximal tubuli of kidney and mucosal epithelium of small intestine. No activity was found in other tissues.

The present method can be regarded as a useful method, not only to examine the specificity of guanase for hepatic diseases, but also to elucidate the physiological significance of guanase in the kidney and small intestine.

Human guanase (guanine deaminase, EC 3.5.4.3) is present mainly in the liver, brain and kidney and there is little or no activity in other tissues. Passanenti (23) was the first to report that guanase appears in the plasma of patients with liver damage. Subsequently, the clinical significance of serum guanase as a liver function test was reported by other investigators (13, 14, 19, 20) and high serum guanase is now considered a specific indicator of hepatocellular damage (7, 10). Moreover, Yamasaki et al. (24), Mihara et al. (21) and Ito et al. (12) reported that the measurement of guanase activity in the donor blood is clinically very important, because the development of post-transfusional non A non B hepatitis can be prevented by such measurement. No histochemical studies of liver guanase, which may be useful in assessing the extent of hepatocellular disease, have been performed as yet. Norstrand et al. (22) demonstrated guanase in the human central nervous system.
system, but no other tissues have been examined to date, and their method has technical shortcomings due to the poor solubility of the substrate (8). We previously reported a modified method of histochemical demonstration of guanase in which guanine is solubilized in bicine buffer (11).

This report describes histochemical distribution of guanase in human tissues using the new method described previously (11).

MATERIALS AND METHOD

Materials
Reagents

Guanine, N,N-bis(2-hydroxyethyl)glycine (bicine), xanthine oxidase (grade III, 19.8 U/ml), nitro tetrazolium blue (NBT) and 5(4)-aminoimidazol-4(5)-carboxamide hydrochloride were purchased from Sigma Chemical Co., U.S.A. Other reagents of the highest available purity were obtained from standard sources.

A bicine buffer was prepared according to our previous report (8, 11).

The substrate for histochemical studies was prepared by mixing 1.0 ml working guanine solution, 0.1 ml xanthine oxidase and 11.0 ml 0.1 M bicine buffer. After incubation at 37°C for 5 min to ascertain that there was no visual change, 1.0 ml NBT (1 mg/ml in 0.1 M bicine buffer) was added.

Tissues

Autopsy samples of macroscopically normal portions of the esophagus, stomach, small intestine, large intestine, liver, gall bladder, common bile duct, pancreas, spleen, kidney, lung, heart muscle and skeletal muscle were obtained as follows: Case I: 60-year-old male, hepatoma, Case II: 66-year-old male, lung cancer, Case III: 53-year-old female, gastric cancer. Autopsy samples of macroscopically abnormal regions of liver were obtained as follows, Case IV: 54-year-old male, hepatoma, Case V: 62 year-old male, metastatic tumor from colon. The times from death to quenching of all autopsy samples were all within 10 hr. A biopsy sample of liver was obtained from Case VI: 29-year-old male, non A non B hepatitis.

Methods

Histochemical demonstration of guanase

Localization of the enzyme in tissue sections is dependent upon the hydrolytic deamination of the substrate guanine to xanthine via guanase, followed by the conversion of the xanthine formed to uric acid by xanthine oxidase. NBT participates in the redox reaction as a hydrogen acceptor, with the resultant deposition of insoluble colored diformazan granules where there is guanase activity.

All samples were snap frozen in liquid nitrogen and cryostat sections, 10 µm thick, were prepared. After fixation with 2.5% glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.4, for 1 hr at room temperature, the sections were rinsed in bicine buffer (0.1 M, pH 7.8) and incubated for 15 min at 37°C with xanthine oxidase to oxidize endogeneous xanthine and hypoxanthine to uric acid. The sections were again rinsed in bicine buffer, transferred to the substrate mixture and
incubated for 1–2 hr at 37°C. After being rinsed in distilled water, sections were allowed to air-dry, and were dehydrated with graded ethanols, transferred to xylene and mounted in a synthetic resin. In order to ensure that the diformazan granules visualized as the final product were attributable solely to guanase activity, the following control procedures were employed:

1) Omission of guanine from the substrate mixture.
2) Omission of xanthine oxidase from the substrate mixture.
3) Heating of sections of liver at 100°C for 10 min prior to fixation.
4) Fixation with 10% formalin for 1 hr.
5) Fixation with acetone for 1 hr.
6) Incubation of sections with 0.001 M 5(4)-aminoimidazol-4(5) carboxamide, a specific and irreversible inhibitor of guanase (15).

As a further control, sections of skeletal muscle, in which no guanase activity was expected, were taken through the complete histochemical procedure, and also through the control manipulations.

**RESULTS**

Control studies

In the results of the control studies, formazan granules were absent from all skeletal muscle sections regardless of substrate used or section pre-treatment. All

<table>
<thead>
<tr>
<th>Tissues</th>
<th>Case I</th>
<th>Case II</th>
<th>Case III</th>
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<tbody>
<tr>
<td>Esophagus</td>
<td>−</td>
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<td>−</td>
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<tr>
<td>Stomach</td>
<td>Cardia</td>
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<td></td>
<td>Body</td>
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<td></td>
<td>Pylorus</td>
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<td>Small intestine</td>
<td>Duodenum</td>
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<td></td>
<td>Jejunum</td>
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<td></td>
<td>Illeum</td>
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<tr>
<td>Large intestine</td>
<td>Colon</td>
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<td></td>
<td>Rectum</td>
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<tr>
<td>Liver</td>
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<tr>
<td>Gall bladder</td>
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<td>Common bile duct</td>
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<tr>
<td>Pancreas</td>
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<tr>
<td>Spleen</td>
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<td>−</td>
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<tr>
<td>Kidney</td>
<td>Bowman’s capsule</td>
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<td></td>
<td>Glomerulus</td>
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<td></td>
<td>Proximal tubule</td>
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<td></td>
<td>Distal tubule</td>
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<td>Lung</td>
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<td>−</td>
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<tr>
<td>Heart muscle</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Skeletal muscle</td>
<td>−</td>
<td>−</td>
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+ or − : with or without diformazan products
control procedures resulted in elimination of the formazan reaction product from
sections of liver.

Distribution of guanase in human tissues

Table 1 shows the histochemical distribution of guanase in human tissues. The formazan reaction could be seen in the small intestine, liver and kidney; however, no formazan deposits could be seen in other tissues.

Distribution of guanase in liver: The distribution of guanase in the liver of Case II, histologically normal, was confined to the cytoplasm of hepatocytes and appeared more intense in areas abutting sinusoids. Portal components showed no activity, but the portal tracts were surrounded by a zone of high guanase content which diminished to a weak reaction in the perivenular regions. All nuclei and membranes showed no formazan granules (Fig. 1).

The guanase reaction in Case III (Fig. 2B) is shown contrasted with section

Fig. 1. Guanase stain of the normal liver (Case II). Guanase activity is shown in the cytoplasm of hepatocytes and absent from nuclei and portal components. A. ×80, B. ×400
Histochemical Demonstration of Guanase

stained with hematoxyline-eosin (H & E) (Fig. 2A), which is included to show the widened portal area with inflammatory cells and fibrous tissue. No formazan deposits could be seen in fibrous tissue or portal veins, however, the cytoplasm of hepatocytes showed a strongly positive formazan reaction.

The formazan deposits in case IV, hepatocellular carcinoma, could irregularly be seen in tumor cells; however, no formazan reaction deposits could be seen in the capsule of tumor (Figs. 3A, B).

The formazan reaction in Case V, metastatic tumor from colon cancer, could not be seen in tumor cells (Figs. 4A, B).

The H & E stain of Case VI, non A non B chronic hepatitis, showed a widened...
portal area with inflammatory cells and piecemeal necrosis (Fig. 5A). The formazan reaction in case VI could not be seen in portal components. In some areas, faint or no formazan reaction could be seen in hepatocytes, and some hepatocyte nuclei showed guanase activity (Figs. 5B, C).

Distribution of guanase in intestine: The guanase reaction in the duodenum of Case II (Fig. 6B) is shown contrasted with section stained with H & E (Fig. 6A), which is induced to show the mucosal epithelium. No formazan deposits could be seen in intestinal tissue, crypts of Lieberkuehn, Brunner's glands, submucosal tissue and mucosal plate. The distribution of guanase in the jejunum (Fig. 7) and ileum was similar to that of the duodenum. Again, only mucosal epithelium showed strong guanase activity, but no guanase reaction could be seen in other areas.

Distribution of guanase in kidney: The guanase reaction could be seen in the proximal tubuli, but no formazan deposits could be seen in the glomeruli, Bowman's capsule and distal tubuli (Fig. 8).
Guanase catalyses the hydrolytic deamination of guanase to xanthine, which in turn is oxidized by xanthine oxidase to uric acid and hydrogen peroxide. Norstrand et al. (22) reported the histochemical demonstration of guanase in the human central nervous system by a method which relies on the hydrolytic deamination of the substrate guanine to xanthine via guanase. A secondary reaction oxidizes xanthine to uric acid, with a concomitant reduction of NBT. However, this method has some shortcomings. Guanine is hardly solubilized in phosphate buffer and so forms fine crystals at room temperature. Therefore, the concentration does not retain the optimum for the enzyme reaction (8). Making use of these findings, we reported a new method for the sensitive and reproducible measurement of serum guanase activity (9). We then considered that the use of

Fig. 4.  H & E stain (A) and guanase stain (B) of the liver with the metastatic tumor cells (adenocarcinoma of colon) (Case V, ×30). The diformazan reaction cannot be seen in the metastatic tumor cells.
bicine buffer should increase the sensitivity of the histochemical demonstration of guanase, and we devised a modification of the method of Norstrand et al. using guanine in bicine buffer as a substrate (11).

All control experiments showed negative results, highlighting the specificity of the final colored reaction product as a result of guanase activity.

Both intracellular and lobular distribution of guanase could be ascertained and we observed an intensely blue formazan reaction product presented within the cytoplasm of hepatocytes throughout the entire lobule. However, in Case VI there were faint formazan reactions in those liver cells supposed to present cellular damage, suggesting some correlation between hepatocellular activity and guanase activity. Portal components, including bile ducts and veins, fibrous tissue and

Fig. 5. H & E stain (A; ×30) and guanase stain (B; ×30, C; ×300) of the liver with chronic hepatitis non A non B type (Case VI). In some areas, hepatocytes show faint color or no color. Some hepatocyte nuclei (▼) show positive guanase activity. Other nuclei (▼) show negative guanase activity.
inflammatory cells also failed to show the formazan reaction.

The activity of guanase in cell fractions obtained by differential centrifugation of rat liver homogenate has been examined by de La Mirande et al. (17) and Bowkiewicz-Surma & Krawczynski (1). The former group found all guanase recoverable in the supernatant fraction—nuclei, microsomes and mitochondria showed no activity. The latter group used tissue from animals which had been anesthetized with ether prior to killing and found the microsomal plus cytoplasmic fraction to contain only 73% of total guanase, nuclei having 15% and mitochondria the remaining 12% of activity. In this study, no guanase reaction product was observed within nuclei in normal liver. These findings are in accord with those of de La Mirande et al., although we cannot exclude the possibility that nuclear and mitochondrial enzyme may have diffused into the cytoplasm. In fact, in cases of non A non B hepatitis nuclei presenting guanase activity or otherwise were identified. This phenomenon of dissociation of guanase activity among nuclei still remains to be elucidated, however, it seems to be an interesting phenomenon.

Fig. 6. H & E stain (A) and guanase stain (B) of the duodenum (Case II, x80). The mucosal epithelium shows strong guanase activity.
in terms of the investigation into the relationship between the pathology of the hepatitis and guanase activity.

Diformazan formation was observed for primary liver tumor, but not for metastatic tumor. Guanase activity is thought to present strong specificity for the former tumor, probably making it possible to make this method available for the differentiation of these tumors.

In terms of the distribution of guanase in human tissues there are many reports in which the distribution has been biochemically examined, though they are not always consistent in their respective results because of different experimental animals and measuring methods (3-5). Guanase is thought to be contained mostly in liver, brain and kidneys, but little in other tissues, and thus there are few reports in which guanase has been examined in other tissues. Levine et al. (18) and Kuzumits et al. (16) identified guanase activity in large and small intestines other than liver, brain and kidneys, though to a small extent, but little activity in

Fig. 7. H & E stain (A) and guanase stain (B) of the jejunum (Case II, ×80). The mucosal epithelium shows strong guanase activity.
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other tissues. The authors’ histochemical results revealed guanase activity in liver cytoplasm to a great extent, and also in kidneys’ proximal tubuli and mucosal epithelium of small intestine to the same extent as in liver. No activity is found in the submucous layer and muscular tunics of the small intestine, so that guanase specific activity is thought to be less in the small intestine than in the liver on the basis of the results as determined on homogenates from all of its layers. Therefore, it can be thought that the present histochemical results the small intestine are not inconsistent with those of Levine et al. and Kuzmits et al. Levine et al. (18) reported the presence of a small amount of guanase activity in the large intestine, as well. However, the authors’ histochemical results revealed no such presence. Though the reason is not yet known, it may be because of the difference in substrate. Histochemical reports on the distribution of guanase in human tissues have concerned

Fig. 8. Guanase stain of the kidney (Case II). Guanase activity is shown in the proximal tubuli. A. ×80, B. ×400
only the results obtained by Norstrand et al. (22) in the human central nervous system, and there are no reports on the distribution of guanase in other tissues. The authors made clear the histochemical distribution of guanase in various human tissues using the modification method of Norstrand et al.

The present method, which is simple and convenient, makes it possible to obtain stable results even for comparatively fresh autopsy specimens, and therefore can be regarded as a useful method not only to examine the specificity of guanase for hepatic diseases but also to elucidate the physiological significance of guanase in the kidney and small intestine.

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REFERENCES