Quantitative Cytochemical Detection of Malignant and Potentially Malignant Cells in the Colon


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ABSTRACT

It was found to be possible to distinguish malignant cells from normal cells by using an oxygen-sensitive tetrazolium salt (neotetrazolium) for the histochemical demonstration of glucose-6-phosphate dehydrogenase activity in cryostat sections of human colon. We have studied 12 cases of established adenocarcinoma of the colon in addition to 4 of ulcerative colitis and 4 of adenomatous polyposis (polyposis coli). In a nitrogen atmosphere the activities of malignant and normal cells were similar. However, after incubation in an atmosphere of pure oxygen, only malignant cells gave a positive reaction after 5 min. Three of the four cases of adenomatous polyposis gave a positive reaction for glucose-6-phosphate dehydrogenase activity in oxygen in a manner similar to that of specimens with severe dysplasia. In general, positive foci were histologically indistinguishable from the neighboring tubuli. However, foci of severely dysplastic epithelium usually showed a positive reaction. Three of the patients eventually developed clear-cut severe dysplasia. The other patient, who showed a negative reaction in oxygen, was diagnosed after 3 years as not suffering from dysplasia. All cases of ulcerative colitis gave a reaction in oxygen comparable with that of normal cells. Therefore, the areas with a positive reaction are considered to be either in the process of malignant transformation or malignant. An explanation for the oxygen insensitivity of cancer cells appeared to be a decrease in the activity of superoxide dismutase (EC 1.15.1.1), as addition of exogenous superoxide dismutase to malignant cells caused a normal reaction. We wish to suggest that this test in combination with the routine histology may be exploited for the diagnosis of polyps in premalignant conditions.

INTRODUCTION

Because the process of carcinogenesis involves a stepwise progression, which often takes over 20 years in humans (1), the prospect of detecting at an early stage those cells that will progress to malignancy is important. Initiated cells often form structures considered to be precancerous, such as polyps or papillomas, some of which will eventually progress to malignancy (2). Therefore a simple test that is able to distinguish such cells at an early stage would be a valuable tool in diagnosis. However, morphological changes often follow the events which cause cells to progress to malignancy (3); thus by the time a cell can be distinguished as malignant by morphological criteria its cancerous nature is already well established.

Changes in the activities of certain enzymes occur at early stages of malignancy (4) and hence are detectable before the morphological changes. One such enzyme is glucose-6-phosphate dehydrogenase (EC 1.1.1.49), the key regulatory enzyme of the pentose shunt pathway that produces both NADPH and the precursors of nucleic acid synthesis (5) which are needed in large amounts by malignant cells.

Demonstration of this enzyme is possible in tissue sections by using a tetrazolium salt coupled to an intermediate electron carrier. Production of an insoluble colored formazan marks the areas of enzyme activity (6). Oxygen present in the immediate reaction area is able to interfere with the production of formazan if either low concentrations of the tetrazolium salts (<5 mM) are used or a tetrazolium salt of high electron potential (e.g., neotetrazolium) is used (7).

It is not sufficient simply to demonstrate glucose-6-phosphate dehydrogenase activity in tissue sections marking the areas of high activity as malignant. High activity may be present in any proliferating cell, malignant, benign, or normal (8). When the histochemical reaction is performed with neotetrazolium in an atmosphere of 100% oxygen, the reaction in normal cells is prevented for 5–15 min, whereas malignant cells show formazan deposition long before this time (8–10). Thus with an incubation time of 5 min in oxygen the histochemical reaction will be prevented in normal cells, whereas malignant cells retain at least a proportion of their activity.

We have studied the effect of oxygen on the histochemical reaction in various samples of colon carcinoma in comparison to normal tissue from the same patients using neotetrazolium as the final electron acceptor. In addition, we have applied the test to ulcerative colitis with severe cellular infiltrate and adenomatous polyps to reveal areas of "potential" malignancy. These areas contained dysplastic cells but could not be classified as malignant by morphological criteria.

SOD (EC 1.15.1.1) has been implemented to affect formazan production in the presence of oxygen during the detection of NADH or NADPH generation in vitro (7, 11). SOD is a protective enzyme that accelerates the dismutation of superoxide radicals which can damage the cell (12). The activity of this enzyme is known to be decreased in malignant cells (13). We therefore investigated the effect of exogenous SOD upon the activity in malignant cells to better understand the cause of oxygen sensitivity of neotetrazolium reduction and the way this is overcome in malignant cells.

MATERIALS AND METHODS

Samples of normal tissue, adenocarcinoma, and ulcerative colitis of the colon were obtained from resected specimens supplied by the Pathology Department, Academic Medical Centre, Amsterdam, The Netherlands. Twelve cases of adenocarcinoma of the colon with the corresponding normal tissue from an area uninvolved in tumor growth and four cases of ulcerative colitis were investigated. In addition, five polyps obtained by endoscopic biopsy were also studied. Hematoxylin and eosin-stained sections of all formalin-fixed samples were used for diagnosis.

The tissue samples were immediately frozen in liquid nitrogen and stored at −80°C before use. Sections 8 μm thick were cut on a motor-driven Bright cryostat fitted with a retracting microtome at a constant low speed to ensure uniformity of section thickness (14). The cabinet

5 The abbreviations used are: SOD, superoxide dismutase; 1-methoxyPMS, 1-methoxyphenazine methosulfate; CAT, catalase; DIC, dicumarol; NT, neotetrazolium; NBT, nitroblue tetrazolium.
temperature was −25°C; the sections were placed on clean glass slides and stored in the cryostat cabinet until use (1 to 2 h). Serial sections to those used for enzyme histochemical studies were also stained with hematoxylin and eosin for reference to all formalin-fixed tissue.

The incubation media for glucose-6-phosphate dehydrogenase consisted of: 50 mM glycyl glycine buffer (BDH Chemicals, Poole, Dorset, United Kingdom) (pH 8.0) or 100 mM phosphate buffer (pH 7.45) containing 20% (20 g/100 ml) polyvinyl alcohol (hot water soluble; average molecular weight 40,000; Sigma Chemical Co., St. Louis, MO) (15), 10 mM glucose 6-phosphate (Boehringer, Mannheim, Federal Republic of Germany); 0.8 mM NADP (Boehringer), 4.5 mM neotetrazolium chloride (Polysciences, Northampton, United Kingdom, or Sigma; purified with chloroform) and 0.67 mM thionine (Merck, Darmstadt, Federal Republic of Germany) as an alternative electron carrier to 1-methoxyPMS (16). The incubation medium for the demonstration of NADPH dehydrogenase was identical but 0.67 mM menadione (sodium salt; Sigma) replaced thionine as the intermediate electron carrier (16, 17). In the course of the investigation the following compounds were added to the basic incubation media either separately or in combination: 1 mM dicumarol (Sigma); SOD (Sigma; from bovine liver, 1600 lU/ml incubation medium); 10 mM sodium azide (Merck). The reaction was performed in a room maintained at 37°C on the average in normal tissue when the incubation was performed in nitrogen (mean, 7.8 min). In oxygen the lag was slightly increased to 1 or 2 min (mean, 1.5 min).

The rate of formazan formation was not seriously affected by the presence of oxygen in the majority of cases (Table 1; Fig. 2a). The residual activity observed in malignant cells was between 30 and over 100% of that in nitrogen (mean, 59%; Table 1; Fig. 1) after 5 min incubation. No significant difference was observed between the reaction rates in malignant cells in oxygen compared to nitrogen. In nitrogen, a linear response was observed up to 7 to 11 min, in oxygen the linear response was usually for a longer period of time (up to 15 min of incubation). No relationship was observed between the degree of differentiation of malignant cells and the lag phase or rate of reaction.

Normal Cells. In nitrogen, the lag phase exhibited by normal cells was below 3 min (mean, 1.0 min), in oxygen this was extended over a range with a mean value of 7.8 min (Table 2; Fig. 2b). The difference between the lag phase for normal cells in nitrogen and in oxygen was significant (P < 0.001). Any reaction observed in normal cells before 5 min had elapsed was always lower than 10% when compared to the reaction in oxygen (mean, 1.2%; P < 0.001 compared to malignant cells; c.f. Tables 1 and 2). Hence normal cells could still easily be distinguished from malignant cells by comparison of the reaction after 5 min in oxygen with that obtained in nitrogen (Fig. 1). The reaction rate at 5 min was always considerably lower in oxygen than nitrogen (P < 0.001; Table 2; Fig. 2b).

Adenomatous Polyps. All cases of adenomatous polyposis were classified independently in serial sections stained with hematoxylin and eosin and were of varying grades. Results obtained with the glucose-6-phosphate dehydrogenase reaction are shown in Table 3 and Fig. 3. Those areas classified as normal colonic tissue all showed no formazan formation in oxygen (negative reaction). Some areas of cells showing a negative reaction were classified by the pathologist as dysplastic, whereas other areas showed a high activity in oxygen (positive reaction; Table 3). These latter areas were classified by the pathologist as severely dysplastic but were morphologically difficult to discriminate from the areas of dysplasia showing a negative reaction on frozen sections (Fig. 3). On the other hand, grading of dysplasia is often subjective when using hematoxylin and eosin-stained sections of formalin-fixed tissue. Positive reactions were typically seen either in areas composed of many glands showing a high reaction and separated from surrounding structures by connective tissue, or in singular glands surrounded by areas displaying very low reaction or no advantage in showing the differences between malignant and normal cells to use glycyl glycine buffer. In human colonic tissue formazan formation was selectively prevented in cancer cells in oxygen for approximately 1.5 ± 1.1 min (SD) (Table 1) and in normal cells in oxygen for approximately 7.5 ± 3.4 min (Table 2; Fig. 1). Of even greater importance was the purity of the neotetrazolium used. Neotetrazolium from Sigma was heavily contaminated with impurities (22) that caused formazan formation in normal tissue in oxygen, hence making it impossible to locate areas of malignant cells. Sigma neotetrazolium therefore had to be purified with chloroform prior to use (22). The batches obtained from Polysciences were found to be pure enough to be used without chloroform treatment and were used for all studies thereafter.

Carcinoma Cells. Carcinoma cells in human colon displayed varying activities of glucose-6-phosphate dehydrogenase (Table 1). The lag phase before any formazan formation was observed in carcinoma cells was typically below 1 min when the reaction was performed in nitrogen (mean, 0.4 min). In oxygen the lag was slightly increased to 1 or 2 min (mean, 1.5 min).

The histochemical method for the detection of glucose-6-phosphate dehydrogenase activity was specific, inasmuch as the control reaction performed by the omission of the coenzyme and substrate from the incubation media (21) reduced the production of formazan to unmeasurable levels. The use of phosphate buffer resulted in a lag phase of 1 or 2 min (mean, 1.5 min).

The use of phosphatase buffer resulted in a lag phase of 4 min on the average in normal tissue when the incubation was performed in oxygen. On the other hand, glycyl glycine buffer gave a lower rate of reaction and a longer lag phase in oxygen in normal tissue (approximately 8 min). Therefore it was a distinct advantage in showing the differences between malignant and normal cells to use glycyl glycine buffer. In human colonic tissue formazan formation was selectively prevented in cancer cells in oxygen for approximately 1.5 ± 1.1 min (SD) (Table 1) and in normal cells in oxygen for approximately 7.5 ± 3.4 min (Table 2; Fig. 1). Of even greater importance was the purity of the neotetrazolium used. Neotetrazolium from Sigma was heavily contaminated with impurities (22) that caused formazan formation in normal tissue in oxygen, hence making it impossible to locate areas of malignant cells. Sigma neotetrazolium therefore had to be purified with chloroform prior to use (22). The batches obtained from Polysciences were found to be pure enough to be used without chloroform treatment and were used for all studies thereafter.
reaction at all in oxygen (Fig. 3b). In nitrogen these activities were very similar.

Cases of Ulcerative Colitis. Table 3 also shows the mean residual activity in epithelial cells affected by ulcerative colitis. None of the cells exhibited any morphological signs of malignancy and reacted in a manner which would classify them as nonmalignant according to the present oxygen sensitivity test. No reaction was seen in the connective tissue or, more importantly, in the cellular infiltrate if thionine was used in the incubation medium. On the other hand, when 1-methoxyPMS was used instead of thionine, a very high reaction was found in inflammatory cells in oxygen and therefore could not be used to indicate areas of malignancy.

Elucidation of the Mechanism of the Oxygen Insensitivity Phenomenon. By replacing 1-methoxyPMS or thionine in the incubation media with menadione, the activity of the enzyme NADPH dehydrogenase is revealed (Fig. 4a). The reaction was specific inasmuch as addition of dicumarol, a specific inhibitor of this enzyme (23), reduced formazan production to low levels. The effect of oxygen upon the residual activity of this enzyme in malignant cells was far greater than the corresponding reaction for glucose-6-phosphate dehydrogenase activity (14% compared with 31%). Addition of dicumarol to the incubation medium for glucose-6-phosphate dehydrogenase resulted in a small but significant increase in the reaction (Fig. 4a).

The effect of exogenous SOD and catalase upon the residual activity of malignant cells in oxygen is shown in Fig. 4b. A decrease from 31% activity to 5% activity was noted; i.e., the malignant cells showed the characteristic residual activity displayed by normal cells in oxygen when exogenous SOD was added to the medium. Addition of azide, an inhibitor of catalase (12), increased the reaction in normal cells in oxygen (not shown). The reaction was dependent upon an intermediate carrier in the medium as it was reduced to low levels when 1-methoxyPMS or thionine was omitted (Fig. 5).
CYTOCHEMICAL DETECTION OF MALIGNANCY IN THE COLON

Fig. 1. Serial cryostat sections (8 μm thick) of adenocarcinoma of the colon, a, hematoxylin and eosin staining of normal (N) and malignant (CA) tissue; b, glucose-6-phosphate dehydrogenase activity after 5 min of incubation using neotetrazolium in an atmosphere of nitrogen. Formazan is deposited in both normal (N) and malignant (CA) tissue; c, as for b, but reaction was performed in an atmosphere of oxygen, showing formazan deposition only in malignant cells (CA). Bar, 100 μm.

DISCUSSION

The oxygen insensitivity test documented here seems to be specific for malignant cells. In normal colonic epithelial cells and in the inflammatory condition of ulcerative colitis where the cells showed no morphological signs of malignancy, a negative reaction was obtained in an oxygen atmosphere (Tables

![Graph](image)

**Fig. 2.** a, kinetic histochemical reaction for glucose-6-phosphate dehydrogenase in cryostat sections of adenocarcinoma of the colon followed on the stage of a Vickers M85a cytophotometer; data shown as nm NADPH formed/mm² tissue against time, showing (Δ) reaction in malignant cells in nitrogen, and (○) reaction in oxygen. No significant difference at 5 min incubation. b, kinetic histochemical reaction for glucose-6-phosphate dehydrogenase followed as for a, in normal colonic epithelial cells. (Δ) reaction in nitrogen; (○) reaction in oxygen. After 5 min of incubation the reactions are significantly different (P < 0.001). Bars, SD.

**Table 3** Residual glucose-6-phosphate dehydrogenase activity (ratio of activity in oxygen and in nitrogen) measured after 5 min incubation in colon polyps and ulcerative colitis

Areas of potential malignancy (residual activity approximately (or >) 30%) were observed in three of the five polyps while all samples of ulcerative colitis proved to be nonmalignant (residual activity <10%).

<table>
<thead>
<tr>
<th>Patient</th>
<th>Area 1 (%)</th>
<th>Area 2 (%)</th>
<th>Area 3 (%)</th>
</tr>
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<tbody>
<tr>
<td>Polyp</td>
<td></td>
<td></td>
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</tr>
<tr>
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</tr>
<tr>
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<td>56</td>
<td>8.7</td>
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</tr>
<tr>
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<td>0</td>
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<tr>
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<td>6.2</td>
<td>2.5</td>
</tr>
<tr>
<td>16-II</td>
<td>23</td>
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<tr>
<td>Ulcerative colitis (mean values: %)</td>
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</tr>
<tr>
<td>17</td>
<td>9</td>
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* Residual activity classifies these areas as possibly malignant.
Fig. 3. Serial cryostat sections (8 μm thick) of a sample of colonic polyp showing (a) hematoxylin and eosin staining; (DC) and (DN) two areas of dysplastic cells. (b) Reaction for glucose-6-phosphate dehydrogenase with neotetrazolium in oxygen after 7 min showing a negative reaction in the DN dysplastic cells and a strong positive reaction in the DC dysplastic cells, suggesting the DC cells to be possibly malignant. Bar, 100 μm.

Cancer cells show an altered pattern of growth and morphological changes such as nuclear pleomorphism, an increase in the nucleus:cytoplasm ratio and abnormal mitotic figures; as such they are easily distinguished from normal cells. It seems therefore that only malignant cells show a positive reaction in oxygen (i.e., residual activity of at least 30%; Table 1), and nonmalignant cells do not show a residual activity of more than 10% (Table 2).

Polyps may eventually progress to malignancy (24, 25) and in the samples of polyps studied here certain areas were oxygen positive (Table 3). These were areas of dysplastic cells that were morphologically indistinguishable from neighboring cells which were also dysplastic. These neighboring cells gave negative reactions in oxygen. The conclusion drawn from this is that cells showing a positive reaction could either be pre-malignant or have a metabolism similar to that of malignant cells. Foci of severely dysplastic epithelium usually showed a positive reaction. It is interesting to note that of two biopsies obtained from the same patient (16-I and 16-II; biopsy II was taken 18 months after biopsy I), biopsy II displayed areas giving positive reactions whereas biopsy I did not. The pathologist diagnosed biopsy 16-I as light to moderate dysplasia, whereas 16-II was diagnosed as severely dysplastic. Three years later the same patient was diagnosed as having familial polyposis. Patients 13, 14, and 16 developed clear-cut severe dysplasia as was shown in biopsies taken 1–4 years later. A biopsy from patient 15 taken after 3 years showed no dysplasia.

The effect of oxygen on the formation of formazan from neotetrazolium may be explained by the reaction scheme suggested below (Reaction A):

\[ \text{NT}^- + \text{O}_2 \rightarrow \text{NT} + \text{O}_2^- + \text{H}^+ \]  
(A)

\[ \text{NBT}^- + \text{O}_2 \rightarrow \text{NBT} + \text{O}_2^- + \text{H}^+ \]  
(B)

Neotetrazolium radicals (NT-H') are oxidized by molecular oxygen to neotetrazolium with the subsequent production of superoxide radicals. A similar reaction scheme for the effect of...
CUMAROL SENSITIVE. The slightly increased reaction is probably due to the inhibition of endogenous electron transport pathways. This evidence suggests that oxygen insensitivity is not mediated by NADPH dehydrogenase but occurs at the level of the neotetrazolium radical. This is also indicated by the fact that oxygen sensitivity in normal cells has been observed while studying succinate dehydrogenase activity in normal rat liver (18), suggesting that oxygen insensitivity in malignant cells is independent of NADPH. Within certain limitations the enzyme used to demonstrate oxygen insensitivity is in itself unimportant.

SOD is involved directly in the inhibition of formazan production, inasmuch as its addition to the incubation medium caused a decreased formation of formazan in malignant cells in oxygen (Fig. 4b). It is known that the activity of the manganese-SOD is absent in carcinoma cells, whereas the activity of copper/zinc-SOD is sometimes lowered (12, 33). The effect of SOD on neotetrazolium-formazan formation cannot be explained in the same way as for nitroblue tetrazolium, where the equilibrium reaction (Reaction B) is affected. Increasing the concentration of neotetrazolium in the incubation medium does not decrease the effect of oxygen (28, 29). Only when all the oxygen has been removed will the reaction proceed (18).

With respect to the initial formation of formazan exhibited by carcinoma cells in an atmosphere of oxygen (the oxygen insensitivity phenomenon), a factor is responsible for the increased reaction in these malignant cells. A comparison of Tables 1 and 2 shows that carcinoma cells may display a rate of reaction similar to that of normal cells in an atmosphere of nitrogen but exhibit a significantly higher rate of reaction than normal cells in oxygen ($P < 0.001$). Without the addition of I-methoxyPMS to the incubation medium the proportion of NADPH utilized for detoxification reactions is revealed (6, 29). These reactions involve the enzymes NADPH cytochrome P-450 reductase and NADPH dehydrogenase. Since NADPH cytochrome P-450 reductase has been indicated as the factor responsible for the oxygen insensitivity phenomenon (30) the activity of this pathway was investigated. Fig. 5 shows that this enzyme accounts for only a small proportion of the overall reaction in both normal and malignant cells (reaction in the absence of PMS). Moreover it is not known as a transformation-linked enzyme (31) and has also been shown to be oxygen sensitive (32). We therefore conclude that this enzyme is not involved in the oxygen insensitivity phenomenon in malignant cells. On the other hand NADPH dehydrogenase is transformation linked (17) but Fig. 4 shows that the reaction for this enzyme is oxygen insensitive only to a limited extent in malignant cells but not to the extent that the reaction for glucose-6-phosphate dehydrogenase activity appears to be. Furthermore the reaction for glucose-6-phosphate dehydrogenase is not dicumarol sensitive. The slightly increased reaction is probably explained with the formation of formazan as shown in Reaction E.

$$\text{Oxygen} \quad \text{Neotetrazolium} \quad \text{Formazan}$$

$$\text{O}_{2} + 2\text{H}^{+} \xrightarrow{\text{SOD}} \text{H}_{2}\text{O}_{2} + \text{O}_{2} \quad (C)$$

$$2\text{H}_{2}\text{O}_{2} \xrightarrow{\text{CAT}} 2\text{H}_{2}\text{O} + \text{O}_{2} \quad (D)$$

$$\text{Addition of azide to the incubation media reduces the lag phase in normal cells by inhibiting catalase, thereby affecting the efficient functioning of SOD. Catalase and SOD have been shown to be mutually supportive to each other (36). Once the initial oxygen has been removed the reaction proceeds unhindered with the formation of formazan as shown in Reaction E.}$$

$$\text{NT} - \text{H}^{+} + \text{NT} - \text{H}^{+} \rightarrow \text{NT} + \text{NT} - \text{H}_{2} \quad \text{(formazan)} \quad (E)$$

If oxygen is recycled in the manner described here, a relatively small proportion of oxygen can exert an effect much greater than the absolute amount alone could account for. This has been documented with regard to fatty acid oxidation, the exaggerated effect of oxygen being explained in a manner similar to that proposed here (37). In conclusion it is stated that the oxygen insensitivity of malignant cells is caused by a decrease in cellular activity of
SOD. This prevents the recycling of molecular oxygen, thus allowing the deposition of formazan within the first 5 min of incubation. Because levels of SOD are decreased in the early stages of malignancy and remain depressed (13), the detection of cancerous cells before morphological parameters become apparent is a possibility as was shown with the four cases of colonic polyps studied here. Therefore, the oxygen insensitivity test could be a useful additional tool for grading of dysplasia.

The present study is based on cytophotometric analysis of the reactions to firmly establish the validity of the test. However, cytophotometry is not an essential tool for the test and microscopic inspection of serial sections after 5 min of incubation in nitrogen and oxygen is sufficient to detect malignant or potentially malignant cells. False positive reactions have not been found thus far in the present study when thionine was used as exogenous electron carrier. Inflammatory cells showed some positive reaction in the presence of oxygen when thionine was replaced by 1-methoxyPMS. Other studies on the basis of the oxygen insensitivity phenomenon (3, 8, 9, 10, 30) did not report false positive findings either.

ACKNOWLEDGMENTS

The authors would like to express their gratitude to Dr. Norman Walfoord for his help in diagnosis of specimens. They would also like to thank the Pathology Department, Academical Medical Centre, for supply of the specimens. Thanks also to J. Peeterse for photographic work and to Professors J. James and P. J. Stoward for making this work possible.

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