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STUDIES ON LIVER CANCER

I. IMMUNOFLUORESCENT STUDIES ON THE LOCALIZATION OF α-FETOPROTEIN IN PATIENTS WITH PRIMARY LIVER CANCER

TETSUZO KODA and SHIGEYUKI ISHIGAMI

Department of Clinical Research, Research Institute for Microbial Diseases, Osaka University, Yamada-kami, Suita, Osaka

SHIZUO TANABE

Department of Bacteriology, Osaka University Medical School, Yamada-kami, Suita, Osaka

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SUMMARY Monospecific antiserum against human α-fetoprotein was prepared from the ascites of a patient with primary liver cancer. Using this antiserum, the localization of α-fetoprotein in tissue specimens at autopsy was investigated by the direct immunofluorescent method.

In liver tissues, specific fluorescence was diffusely observed in the cytoplasm and in part of the nucleus (probably the nucleolus) of cancer cells. In kidney tissues, in the absence metastasis and bleeding, strong specific fluorescence was found in the cytoplasm of glomerular cells, and also in epithelial cells of the renal tubules. Moreover, cells in the sediment of urine showed specific fluorescence.

INTRODUCTION

In 1964, Tatarinov reported the occurrence of α-fetoprotein in the sera of patients with primary liver cancer. Subsequently, the significance of α-fetoprotein in the diagnosis of primary liver cancer has been widely recognized (Abelev et al., 1967; Uriel et al., 1967; Masseyeff et al., 1968).

Antiserum against α-fetoprotein used in previous studies was obtained from human fetal serum or cord blood serum. In the present work monospecific antiserum was prepared from the ascites of a patient with primary liver cancer. This antiserum was used to test for the presence of α-fetoprotein in the serum, ascites and urine of patients with primary liver cancer. The site of production of α-fetoprotein is unknown, although Abelev et al. (1967) reported that α-fetoprotein is specifically synthesized by malignant liver cancer cells. Immunofluorescent studies have been carried out by several investigators to clarify this problem, but all previous studies were done by an indirect method. The present antiserum was highly purified to avoid non-specific fluorescence and was used for direct immunofluorescent studies on various tissues obtained at

autopsy, and on the sediment of urine.

MATERIALS AND METHODS

1. *Specimens*

The materials examined were 25 specimens of serum, 3 specimens of ascites, 3 specimens of urine and 2 specimens of sediment of urine obtained from patients with primary live cancer, and 3 specimens each of liver and kindey obtained at autopsy. As controls, a large number of sera from healthy adults, pregnant women, umbilical cord sera and sera of patients with various other diseases were examined.

2. *Isolation of human α-fetoprotein and preparation of rabbit antiserum against α-fetoprotein*

The method used is summarized in Fig. 1. The ascites of patients with primary liver cancer were used as starting material. Ammonium sulfate was added to the ascites to make a 50% saturation. The mixture was allowed to stand at room temperature for 3 hr., and then centrifuged at 28,000 rev/min for 30 min at 4 C using a Beckman L type ultracentrifuge. The sediment contained no α-fetoprotein (Fig. 2), so it was discarded. Ammonium sulfate was added to the supernatant to make a 75% saturation. The mixture was allowed to stand at room temperature and then centrifuged as before. Fig. 2 shows that the precipitate contained α-fetoprotein. It was washed 3 times with cold 75% saturated ammonium sulfate solution and then dissolved in distilled water and dialyzed for 48 hr. against phosphate buffered saline, pH 7.2, with frequent changes of the outer solution. Then it was submitted to agar zone electrophoresis. The area containing α_1-globulin was cut out, and the fluid obtained by freezing and thawing the agar matrix was concentrated to a suitable volume by negative pressure ultrafiltration and used as the crude α-fetoprotein preparation. This crude fraction, containing a high level of α-fetoprotein, was injected intramuscularly into rabbits with Freund's complete adjuvant. Five injections were given at two week intervals. One week after the final injection, 20 ml of blood were drawn. As shown in Fig. 3, this antiserum contained at least 4 antibodies against proteins other than α-fetoprotein. To remove these other antibodies, this antiserum was absorbed with immunoadsorbent, glutaraldehyde-gelated human serum containing no α-fetoprotein, by the method of Avrameas (1969). The absorption procedure was repeated until the preparation gave no precipitation line against normal human serum. Generally, one or two absorption treatments were sufficient.

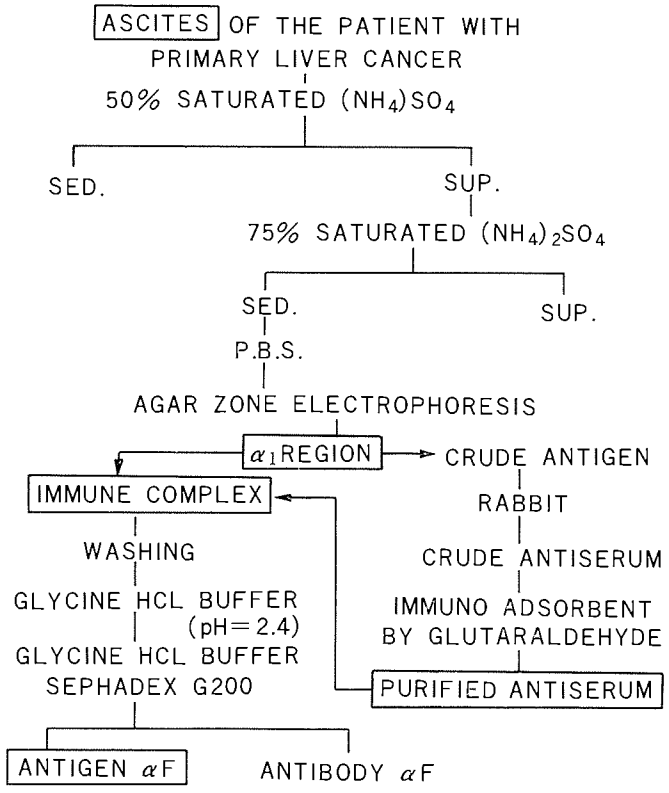

FIGURE 1. *Isolation of human α-fetoprotein and preparation of rabbit antiserum.*

3. *Detection of α-fetoprotein*

The immunodiffusion test (Ouchterlony's method) and microimmunoelectrophoresis were used.

4. *Quantitative determination of α-fetoprotein*

The single radial immunodiffusion method was used. For this method, a highly purified preparation of α-feto-

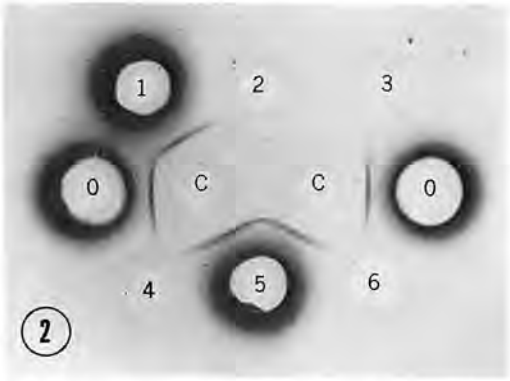

FIGURE 2. *Immunodiffusion picture of various fractions obtained with $(NH_4)_2SO_4$.*

O: Ascites from a patient with primary liver cancer.

Supernatant fluid on 1) 50% saturation, 2) 75% saturation and 3) 100% saturation with $(NH_4)_2SO_4$.

Precipitate on 4) 50% saturation, 5) 75% saturation and 6) 100% saturation with $(NH_4)_2SO_4$.

C: purified antiserum against human α-fetoprotein.

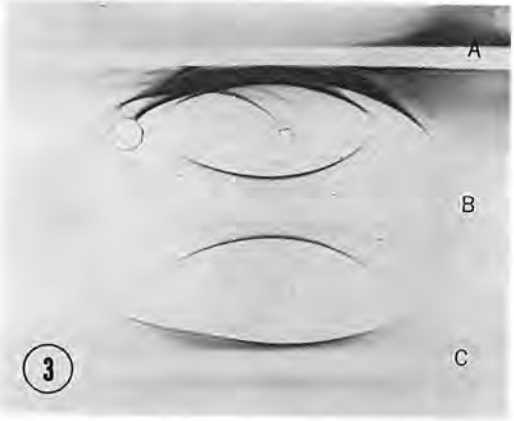

FIGURE 3. *Comparison of immunoelectophoresis of original and pruified antisera against human α-fetoprotein.*

A: Original antiserum against human α-fetoprotein.

B: Purified antiserum against human α-fetoprotein.

C: Antibodies other than that against human α-fetoprotein were isolated with glycine hydrochloride buffer solution.

Wells: All wells contained serum of a patient with primary liver cancer.

protein in which the protein content is known exactly should be used. Therefore, α-fetoprotein was purified by the method shown in Fig. 1. An immune complex was made from purified

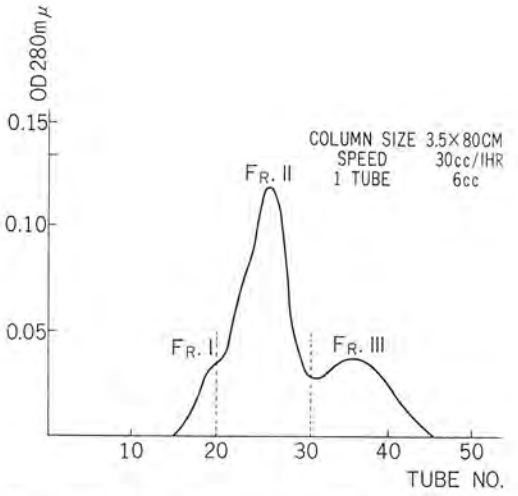

FIGURE 4. *Protein concentration curve obtained by gel filtration on Sephadex G-200.*

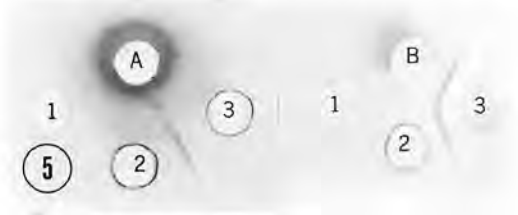

FIGURE 5. *Immunodiffusion pictures of the various fractions obtained by gel filtration on Sephadex G-200.*

1: Fraction 1.

2: Fraction 2.

3: Fraction 3.

A: Antiserum against normal human whole serum.

B: Purified antiserum against human α-fetoprotein.

antiserum and the crude α-fetoprotein fraction. This complex was washed several times with cold phosphate buffered saline, pH 7.0. It was dissociated with glycine hydrochloride buffer, pH 2.4 and then subjected to gel filtration with Sephadex G-200 using the same buffer. As shown in Fig. 4, three peaks of protein were obtained, Fig. 5 shows that the second fraction contained only antibody against α-fetoprotein and the third fraction contained only purified human α-fetoprotein, without any other human serum protein.

The protein content of this purified α-fetoprotein was measured by the micro-Kjeldahl method. This was used as the standard antigen content of α-

fetoprotein together with purified antiserum in the quantitative single radial immunodiffusion method by Fahey (1965).

5. *Localization of α-fetoprotein*

The direct immunofluorescent antibody method was used. Labelled antiserum was prepared by the method of Kawamura (1969). Namely, globulin was separated from rabbit antiserum against human α-fetoprotein by adding ammonium sulfate and was conjugated with fluorescent isothiocyanate.

Portions of liver and kidney obtained at autopsy were kept at −70 C until the fluorescent antibody was applied, and other portions were fixed with 10% formalin solution. For immunofluorescent studies, each tissue was treated with n-Hexane in a vessel containing dry ice acetone and frozen sections of 4–6 μ thickness were prepared in a cryostat at −40 C. The frozen sections were fixed in acetone at −20 C for 15 min and dried in air. Then fluorescent antibody was put on the top of the frozen sections and they were incubated in a moist chamber at 37 C for 1 hr. Then they were washed several times with phosphate buffered saline to remove unreacted reagents, mounted in glycerol buffer solution and examined under a Nikon fluorescent microscope. Histological preparations were stained with haematoxylin and eosin.

RESULTS

1. *Detection of α-fetoprotein*

α-Fetoprotein could be detected in serum and ascites without concentrating the samples, but for its detection in urine, specimens were concentrated 85–200 times. The precipitation lines obtained by the immunodiffusion method are shown in Fig. 6.

α-Fetoprotein was detected in 19 of 25 serum specimens from cases of primary liver cancer (76%), and in all 38 specimens of cord blood

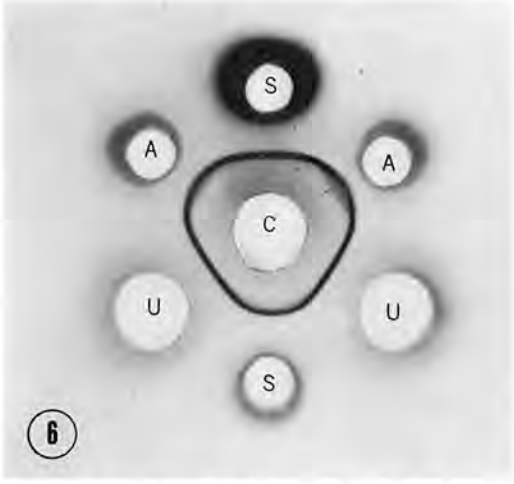

FIGURE 6. *Comparison of immunodiffusion pictures of α-fetoprotein in serum, ascites and urine of a patient with primary liver cancer.*
S: Serum. A: Ascites. U: Urine (concentrated 135 times) C: Purified antiserum against human α-fetoprotein.

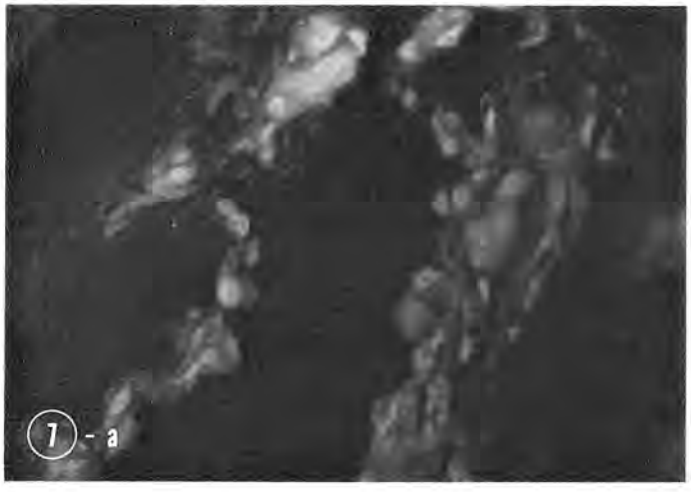

FIGURE 7. *Immunofluorescent picture of the liver from a patient with primary liver cancer obtained at autopsy.*
a) Magnificiation: ×200
b) Magnification: ×400

sera. It was not detected in 140 control sera of normal adults, 10 sera of pregnant women, 11 sera of patients with metastatic liver cancer, 16 sera of patients with liver cirrhosis or 56 sera of patients with various other diseases. (Table 1).

2. *Quantitative determination of α-fetoprotein*

The concentration of α-fetoprotein in the serum was found 130.0–5.80 mg/dl in 10 patients with primary liver cancer, 15.0–2.0 mg/dl in the ascites in 3 cases and 0.33–0.12 mg/dl in the urine in 3 cases (Table 2).

TABLE 2. *Quantitative determination of α-fetoprotein in biological fluids from patients with primary liver cancer*

Material	Number of patient	α-Fetoprotein concentration
Serum	10	130.0 —5.80 mg/dl
Ascites	3	15.0 —2.0 mg/dl
Urine	3	0.33—0.12 mg/dl

TABLE 1. *Detection of α-fetoprotein in sera*

Material	Number of patient	α-Fetoprotein	
		Positive	Negative
Primary liver cancer	25	19 (76%)	6
Cord blood serum	38	38 (100%)	0
Healthy adult	140	0	140
Pregnant woman	10	0	10
Metastatic liver cancer	11	0	11
Cancer of the esophagus	3	0	3
Cancer of the stomach	6	0	6
Cancer of the lung	2	0	2
Other malignant tumors	13	0	13
Hepatitis	32	0	32
Cirrhosis of the liver	16	0	10

3. *Localization of α-fetoprotein*

Autopsy was carried out on 3 cases whose sera had given a positive α-fetoprotein reaction. The livers of these cases weighed 5.3–2.6 kg, and all were confirmed histopathologically as cases of primary hepatocellular carcinoma (trabecular type) with liver cirrhosis but without metastasis to bone marrow, stomach, lung, kidney, spleen or other organs and tissues (Table 3).

Localization of α-fetoprotein was investigated by the direct immunofluorescent method. In liver tissues, as shown in Fig. 7, specific fluorescence was diffusely seen in the cytoplasm and part of the nucleus (probably nucleolus) of

TABLE 3. *Clinical and laboratory findings of autopsied cases*

Case	A.S. 53 y. ♂	T.T. 46 y. ♂	S.S. 75 y. ♂
Onset	June, '70	July, '70	December, '70
Main physical sign			
Liver swelling (below the costal margin)	ca. 3.6 cm	ca. 9.0 cm	ca. 6.0 cm
Ascites	(卄)	(卄)	(−)
Main laboratory findings			
Total bilirubin (mg/dl)	3.9	70.0	5.4
S-GOT (u)	650	1640	160
S-GPT (u)	150	260	128
Alkaline phosphatase (u)	31	40	94
Urine			
Protein	−	−	−
Sugar	−	−	−
Urobilinogen	+	卄	卅
Bilirubin	+	+	卄
Serum protein (g/dl)	8.7	6.4	6.5
A/G ratio	0.60	1.18	0.57
α_1 globulin (g/dl)	0.67	0.43	0.64
Orosomucoid (g/dl)	0.250	0.096	0.140
α-fetoprotein			
Serum (mg/dl)	125.0	90.0	30.0
Ascites (mg/dl)	15.0	15.0	—
Urine (mg/dl)	0.33	0.33	0.12
Findings at autopsy			
Liver weight (kg)	2.6	5.3	3.2
Ascites	(卄)	(卄)	(−)
Histopathology	Primary liver cancer with liver cirrhosis (trabecular type)		
Metastasis to other organs and tissues	(−)	(−)	(−)

cancer cells. No specific fluorescence was observed in non-cancerous cells. In contrast, no specific fluorescence was noted in metastatic cancer cells in the liver or in liver cells of cases of hepatitis or liver cirrhosis obtained by biopsy or autopoy so far as examined. In kidney tissues, as shown in Figs. 8 and 9, strong specific fluorescence was found in the cytoplasm of glomerular cells and also in epithelial cells of renal tubules.

From the findings in the kidney and urine, the sediment of urine specimens of patients with primary liver cancer whose sera showed a positive α-fetoprotein reaction were examined by the direct immunofluorescent method. As shown in Fig. 10, specific fluorescence was observed in cells of the sediment of urine. These cells could not be identified exactly, but may be epithelial cells of renal tubules. No protein was detected in the urine by routine laboratory tests in the clinical course of the disease and neither metastasis nor bleeding nests were found in the urogenital system at autopsy.

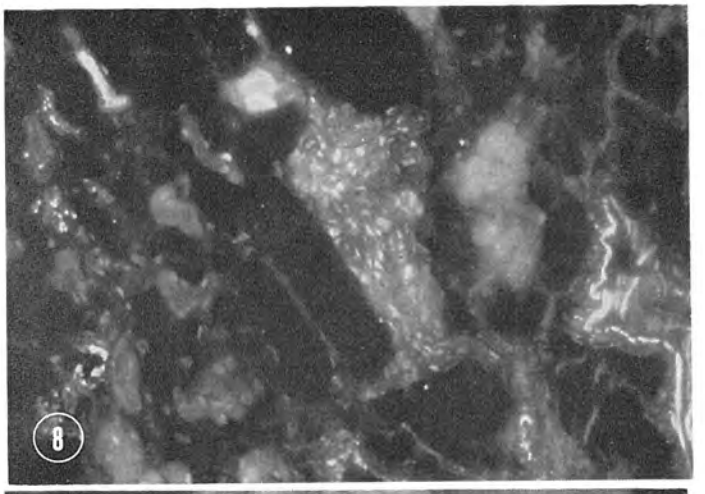

FIGURE 8. *Immunofluorescent picture of kidney tissue of a patient with primary liver cancer obtained at autopsy.*
Magnification: ×200

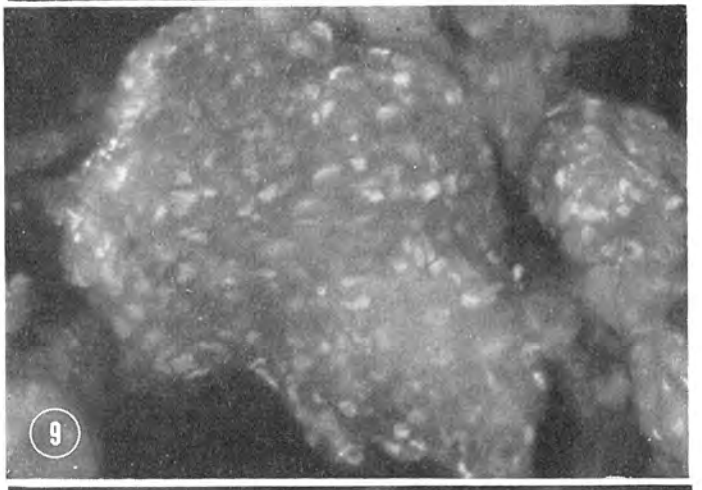

FIGURE 9. *Immunofluorescent picture of glomeruli of the kidney of a patient with primary liver cancer obtained at autopsy.*
Magnification: ×400

FIGURE 10. *Immunofluorescent picture of the sediment of urine from a patient with primary liver cancer.*
Magnification: ×400

DISCUSSION

In almost all previous work, fetal material (serum or extract) and sera from patients with primary liver cancer, because they have high α-fetoprotein contents, have been used as immunogens for preparation of antiserum against α-fetoprotein. However, the supply of fetal materials is restricted and it is also very difficult to obtain a large quantity of blood from a patient with primary liver cancer.

So, as recommended by Uriel et al. (1967), we used ascites of patients with primary liver cancer as a source of immunogen.

When untreated ascites was injected into rabbits as an immunogen, no antibody was evoked against α-fetoprotein although many antibodies were produced against other proteins. The same phenomenon was observed using cord blood sera or sera of patients with primary liver cancer. The reason for this is not known, but may be because of the low content of α-fetoprotein in the immunogen relative to the contents of other proteins. Therefore, we obtained the α_1 globulin fraction of ascites by ammonium sulfate precipitation and zone electrophoresis on agar gel. This fraction was concentrated by negative pressure ultrafiltration and injected into rabbits. The α-fetoprotein contents of this immunogen, measured by the single radial immunodiffusion method, was calculated as 2 mg per ml, so each rabbit was immunized with 10 mg of α-fetoprotein.

Recently, Rappe et al. (1971) reported the partial purification of α-fetoprotein from ascites. They found the α-fetoprotein in the supernatant after 60% ammonium sulfate precipitation and purified by stepwise DEAE-cellulose chromatography. They immunized rabbits with 15 mg of α-fetoprotein and evoked 4 or 5 antibodies at least against proteins other than α-fetoprotein. Our method is easier and simpler than that described by Rappe et al.

Furthermore, using cord blood serum as starting material, we found that the α_1-globulin obtained by this method could also be used as crude immunogen.

Using this antiserum, we examined the presence of α-fetoprotein in the sera, ascites and urine samples of patients with primary liver cancer and various other diseases. Our results were almost the same as those reported by others. But we detected α-fetoprotein in the urine after concentrating the latter 85–200 times, whereas Kithier et al. (1966) reported finding it in urine which had been concentrated 500 times.

The localization of α-fetoprotein in tissue sections obtained at autopsy from patients with primary liver cancer was also investigated. Previously, specific fluorescence has been observed in the cytoplasm of cancer cells using an indirect immunofluorescent method. This paper is the first report of use of a direct immunofluorescent method.

In liver tissues, specific fluorescence was diffusely seen in the cytoplasm and part of the nucleus (probably the nucleolus) of cancer cells. No specific fluorescence was noted in non-cancerous cells. These are important findings, suggesting that α-fetoprotein is synthesized in cancer cells.

In kidney tissues, strong specific fluorescence was found in the cytoplasm of glomerular cells and also in epithelial cells of renal tubules. Furthermore, cells (probably, epithelial cell of renal tubules) in the urine of patients with primary liver cancer (the serum gave a positive reaction for α-fetoprotein) showed specific fluorescence.

There are no previous reports of finding α-fetoprotein in the kidney or the sediment of urine. Further studies are now in progress on this in our laboratory.

ACKNOWLEDGMENTS

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