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Expression of HLA Class I Antigens in Sporadic Adenomas and Histologically Normal Mucosa of the Colon[1]

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ABSTRACT

The loss of HLA antigens by neoplastic cells is considered important for tumor growth and metastasis, inasmuch as it may allow tumors to escape immune surveillance. We have observed reduced expression of HLA antigens in sporadic colon cancer and adenomas from familial adenomatous polyposis patients. We now studied the expression of HLA class I antigens in patients with sporadic adenomas, which are precursors of colorectal cancer. Expression of HLA class I antigens was studied by immunohistochemistry in (*a*) sporadic colon adenomas, (*b*) histologically normal mucosa distant from the adenomas, (*c*) histologically normal colonic mucosa from patients with history of sporadic colon adenomas, and (*d*) colonic mucosa from normal subjects. HLA class I antigen expression was moderately reduced in 56% and severely reduced in 44% of the adenomas; this reduction was significant when compared to controls ($P < 0.0001$). The reduction of HLA class I expression in adenomas was related to the grade of dysplasia of the adenomas. HLA class I expression of normal appearing mucosa was decreased in 76% of patients with adenoma ($P < 0.0001$) and in 54% of patients with history of adenoma ($P < 0.005$) compared to normal controls. These changes were antigen specific, inasmuch as the expression of carcinoembryonic antigen, a surface antigen, was not affected. Our findings suggest that reduced HLA class I expression is an early event in the cell transformation process from normal to neoplastic state, preceding in many cases the onset of histological changes. HLA class I could be potentially used as a premalignant marker in the colon.

INTRODUCTION

Considerable evidence has been accumulated over the past decade on the importance of the complex interactions between tumor cells and the immune system (1, 2). The major histocompatibility complex, referred to as HLA in humans, plays an important role in tumor immunosurveillance (3). HLA class I molecules, which control the recognition of foreign antigens and graft rejection, are transmembrane glycoproteins composed of a heavy chain, encoded at the A, B, C, and other loci in the major histocompatibility complex region of chromosome 6, and a light chain (β_2-microglobulin) encoded in chromosome 17 (4). HLA class I molecules are required for the presentation of tumor neoantigens to cytotoxic T-lymphocytes. There is evidence that tumor cells with reduced expression or lack of such antigens could evade an immune response and be selected for tumor progression (1).

The loss or reduced expression of HLA class I in colorectal cancer has been shown to be a frequent event by us and others (5–9). For example, the expression of HLA class I antigens in colon cancer was dramatically reduced compared to controls, being undetectable in 28%, diminished in 68%, and normal in only 4% of the tumors (8). Recently, we also reported reduction of HLA antigens in adenomas and histologically normal mucosa from patients with familial adenomatous polyposis (10), a rare inherited syndrome characterized by the development of multiple polyps in the colon, which, if not removed, eventually develop into cancer (11).

Characterization of the relationship between changes in the HLA phenotype and transformation of cells may contribute to our understanding of progression of colorectal adenoma to carcinoma and shed light on the mechanisms by which tumor cells at an early stage escape immune destruction. It was, therefore, important to examine whether sporadic colon adenomas, representing the great majority of colon adenomas, and histologically normal mucosa distant from the adenomas display altered HLA class I antigen expression.

In this paper we report the findings from our study of the expression of HLA class I antigens in sporadic adenomas and normal appearing mucosa from patients with synchronous adenomas and also patients with history of adenomas.

PATIENTS AND METHODS

Patients. Included in the study were 110 biopsy specimens from 84 patients (76 male, 8 female; mean age, 66 years) who were colonoscoped at the Veterans Administration Hospital, Martinez, CA, between January and July 1991. Presenting symptoms and past medical history were carefully reviewed in all patients.

The following groups of patients were studied. (*a*) Patients with one or more adenomas: 23 were male and 6 female; their mean age was 69 years (range, 34–87 years). Five of the adenomas were located in the rectum, 12 in the left colon, and 17 in the right colon. In 18 of these patients paired histologically normal mucosa was also available. (*b*) Patients with adenoma(s) from whom only normal mucosa was available. In these patients, for technical reasons the adenomas were not made available for this study; histologically normal mucosa distant from the adenoma was obtained. All 20 were male; their mean age was 67 years (range, 53–81 years) (*c*) Patients with history of colonic adenoma(s): 11 male patients (mean age, 70 years; range, 46–80 years) with personal history of adenoma, and no abnormal findings during surveillance colonoscopy. Histologically normal colonic mucosa was obtained by endoscopic biopsy. (*d*) Normal controls: 24 subjects served as controls (21 male, 3 female; mean age, 62 years; range, 38–75 years). These individuals, evaluated for irritable bowel syndrome, had a normal colonoscopy and no personal or family history of colonic neoplasia.

The diagnosis of adenoma was established in all cases histologically. Adenomas were classified according to the highest grade of dysplasia observed, even if it was present only in a restricted area. In 14 of the adenomas the dysplasia was mild, in 12 moderate, and in 8 severe. The specimens obtained at a distance from the adenomas from patients with history of adenomas, as well as those obtained from subjects with no apparent colonic disease, were all histologically normal.

As soon as the fresh tissue specimen was obtained endoscopically, it was embedded in Optimal Cutting Temperature compound (Miles Scientific, Naperville, IL), frozen immediately in *n*-hexane precooled at −70°C, and stored at −70°C until used.

The study was approved by the Committee for Human Rights in Research.

Immunohistochemistry. Tissue sections (4 μm) mounted on pretreated microscope slides were stained by indirect immuno-peroxidase. Briefly, after equilibration to room temperature for 30 min, samples were fixed in acetone for 5 min and then incubated for 15 min in 0.9% H_2O_2 in methanol. Rehydration was accomplished by three washes in 95% alcohol followed by water,

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before transferring the specimens to PBS.[3] A 30-min incubation in horse serum (Vector Laboratories, Burlingame, CA) that was diluted 1:10 in PBS containing 2% bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was followed by addition of the primary monoclonal antibody. The samples were then incubated in a humidified chamber overnight at 4°C. This was followed by incubation with the secondary biotinylated antibody (horse anti-mouse) for 60 min. The avidin-peroxidase complex (Vector Laboratories) was added for 30 min, followed by the addition of 3,3'-diaminobenzidine tetrachloride (grade II; Sigma), the final coloring agent. Between each antibody treatment the slides were washed three times with PBS. Tissue was counterstained with hematoxylin and examined under the microscope.

Antibodies. Two monoclonal antibodies were used: for HLA class I antigens, clone W6/32 (IgG2a) recognizing shared determinants of HLA-A, -B, and -C (Sera-lab, Westbury, NY); and for CEA a polyclonal antibody recognizing a polymorphic CEA epitope (Dako Corporation, Carpenteria, CA).

Controls. Normal colon mucosa served as positive control for HLA class I antigens. Nonspecific immunoglobulin of the same class and subclass as the primary antibody (Becton Dickinson, San Jose, CA) and at the same concentration was used as a negative control. The expression of CEA was assessed as an additional control of the expression of cell surface molecules.

Evaluation. Both the adenomatous and normal mucosa were evaluated according to the following scale: 4+, all glands stain strongly, *i.e.*, as strongly as normal control tissue processed simultaneously; 3+, all glands stain but their intensity is less than maximal without being "trace" (=category 1+) or <75% of epithelial cells stain; 2+, stained tumor cells between 25% and 75%; 1+, up to 25% of cells staining or trace staining of all or most cells; 0, no glands stain positively. Samples were rated blindly by two pathologists; their evaluations were in excellent agreement.

Statistical Methods. The Wilcoxon signed rank test, the Kruskal-Wallis test, and the Mann-Whitney test were used to compare the frequency of expression of HLA antigens in the various groups of samples that we studied.

RESULTS

Table 1 and Fig. 1 summarize the results of HLA class I antigen expression in both adenomas and histologically normal colonic mucosa. The normal mucosa samples were not only from subjects with a synchronous adenoma but also from patients with a history of adenoma (and no synchronous adenoma) as well as normal subjects. Colonic mucosa from normal subjects expressed HLA class I antigens strongly: in 88% of the patients it was maximal (4+ in our scoring scale) (Fig. 2), and in the remaining 12% it was just below maximal (3+).

HLA class I antigen expression was decreased dramatically in adenomas (Fig. 2). In 44% of the cases the expression of HLA class I antigens was severely reduced or not detectable (1+ or 0), whereas in the remaining 56% it showed moderate reduction (3+ in 6 and 2+ in 13). No adenoma displayed normal expression of class I antigens. Compared to normal controls the HLA class I expression in adenomas was significantly reduced ($P < 0.0001$). The expression of HLA class I antigens in adenomas was also significantly reduced when compared to normal appearing mucosa from patients with either a synchronous adenoma or a history of adenoma ($P < 0.001$).

HLA class I antigen expression was reduced in normal appearing mucosa from patients with a synchronous colonic adenoma compared to colonic mucosa from normal subjects (Fig. 2). In 71% of them this reduction was moderate (2+ or 3+) and in one case it was severe (1+). In the remaining 24% of the samples HLA class I expression was normal. This reduction was statistically significant ($P < 0.0001$). Reduced HLA class I expression was also found in normal appearing mucosa from patients with only positive history of colonic adenoma. In 54% of the cases it was moderately reduced while in 46% it was normal ($P < 0.005$). The difference in the expression of HLA class I antigens between the two groups of histologically normal mucosa was not statistically significant.

In patients from whom both adenoma and normal mucosa samples were available, the HLA class I antigen expression in the adenomas was significantly lower ($P < 0.0001$) compared to that of the normal appearing mucosa distant from the adenoma. The magnitude of the reduction in HLA class I antigen expression in adenomas was not paralleled by that in the corresponding (paired) histologically normal mucosa.

In adenomas there was a significant association between the expression of HLA class I antigens and the degree of dysplasia ($P < 0.001$) (Table 2; Fig. 3). Due to the small sample size it is difficult to formally determine how the three dysplasia grades compare to one another with respect to HLA class I antigen expression, but further analysis suggests that there was greater expression in the group with mild dysplasia than in the group with severe dysplasia. There was no correlation between the location of the adenoma in the colon and HLA class I antigen expression.

Patients with two or more adenomas had a more pronounced reduction of HLA class I antigen expression in their histologically normal mucosa compared to that in patients with one adenoma. However, this difference did not reach statistical significance (Table 3).

Table 1 *HLA class I expression in adenomas and normal mucosa*

Immunoperoxidase scoring as in "Materials and Methods." In the first 18 patients with adenoma, samples of histologically normal mucosa were also available; from the remaining, samples of either the adenoma or histologically normal mucosa were available ("unpaired samples"). A/S, age/sex.

Adenoma[a]		Histologically normal mucosa					
		Synchronous adenoma[b]	History of adenoma[c]		Normal[d]	
A/S	HLA	HLA	A/S	HLA	A/S	HLA
70M	2+	3+	75M	2+	47F	4+
62M	1+	2+	78M	2+		4+
71M	1+	3+	72M	4+	71M	3+
68M	1+	2+	79M	3+	53M	4+
64M	2+	3+	59M	4+	61M	4+
68M	1+	3+	74M	3+	68M	4+
76M	0+	2+	61M	4+	63M	4+
	2+		80M	3+	59M	4+
69M	1+	2+	79M	4+	70M	4+
51M	1+	4+	46M	3+	60M	4+
	2+		70M	4+	75M	4+
	3+				71M	3+
71M	2+	3+			71F	4+
73M	1+	4+			42M	4+
62M	2+	3+			38M	4+
63M	2+	3+			57M	4+
69M	2+	3+			74M	3+
80F	2+	2+			43M	4+
54M	0+	3+			64M	4+
77F	1+	3+			72M	4+
68M	3+	3+			75M	4+
					51M	4+
					70M	4+
					62M	4+
					62M	4+
Unpaired Samples						
71M	3+	69M	3+			
83M	2+	80M	4+			
	3+	70M	2+			
62M	3+	53M	3+			
81F	2+	73M	2+			
58F	3+	68M	4+			
82F	1+	70M	4+			
34F	1+	55M	2+			
86M	2+	60M	1+			
70M	1+	81M	3+			
81M	2+	70M	3+			
87M	0+	63M	4+			
	1+	52M	4+			
		79M	3+			
		53M	2+			
		71M	4+			
		66M	3+			
		75M	2+			
			3+			
		67M	3+			
			4+			
		62M	4+			

[a-d] *a versus b*, $P < 0.001$. a versus c, $P < 0.001$; a versus d, $P < 0.0001$; b versus c, $P = 0.23$; *b versus d*, $P < 0.0001$; *c versus d*, $P < 0.005$ (Kruskal-Wallis test).

[3] The abbreviations used are: PBS, phosphate buffered saline; CEA, carcinoembryonic antigen.

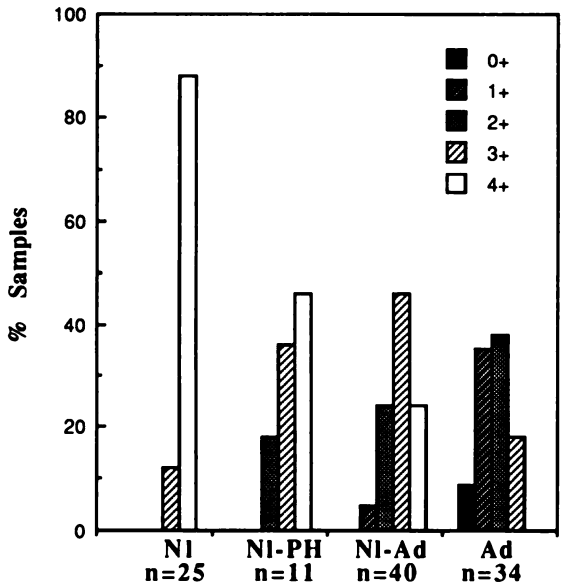

Fig. 1. HLA class I antigen expression in patients with adenoma and normal subjects. *Nl*, normal subjects; *Nl-PH*, normal mucosa from patients with history of adenoma; *Nl-Ad*, normal mucosa from patients with synchronous adenoma; *Ad*, adenoma. Immunoperoxidase scoring as in "Materials and Methods."

In colonic glands from normal subjects the staining was uniformly strong. In patients with adenomas, or history of adenomas, the staining tended to be heterogeneous in both the normal and the adenomatous glands (Fig. 2). In any given polyp the intensity of staining of the adenomatous glands for class I antigens decreased as the degree of dysplasia increased. Occasionally, different staining patterns were observed in areas with the same degree of dysplasia.

The expression of CEA was determined in all groups of tissues studied as a control of the expression of cell surface molecules. There was no difference in the expression of CEA in any of them, being always maximal (4+).

DISCUSSION

The data presented here demonstrate that in patients with sporadic adenomas the expression of HLA class I antigens in colonocytes was reduced compared to that of colon tissue from normal individuals. This reduction was apparent in both adenomatous tissues and histologically normal mucosa. That the levels of CEA, a cell surface antigen, were not altered in any of these tissue samples indicates that the observed reduction is specific for the HLA antigens and not an artifact of the detection method or a result of a nonspecific alteration in membrane proteins.

Loss of HLA class I loci has been reported in the past (7, 12), although others did not find reduction (9). In all these studies the numbers of the adenomas studied were small and did not make any reference to the degree of dysplasia of the adenomas.

The reduction in HLA class I antigen expression involved all stages of the adenoma-carcinoma sequence. Besides adenomas, it also included histologically normal mucosa from patients with a synchronous adenoma distant from the adenoma and histologically normal mucosa in patients who had their polyps removed in the past. Our data on HLA expression in colon cancer (8) revealed that HLA class I antigens were not detectable in 28% of the cases of colon cancer compared to 9% in adenomas, and their expression was severely reduced (1+) in 45% *versus* 35%, respectively. These findings, therefore, indicate that HLA class I underexpression is closely associated with the neoplastic process.

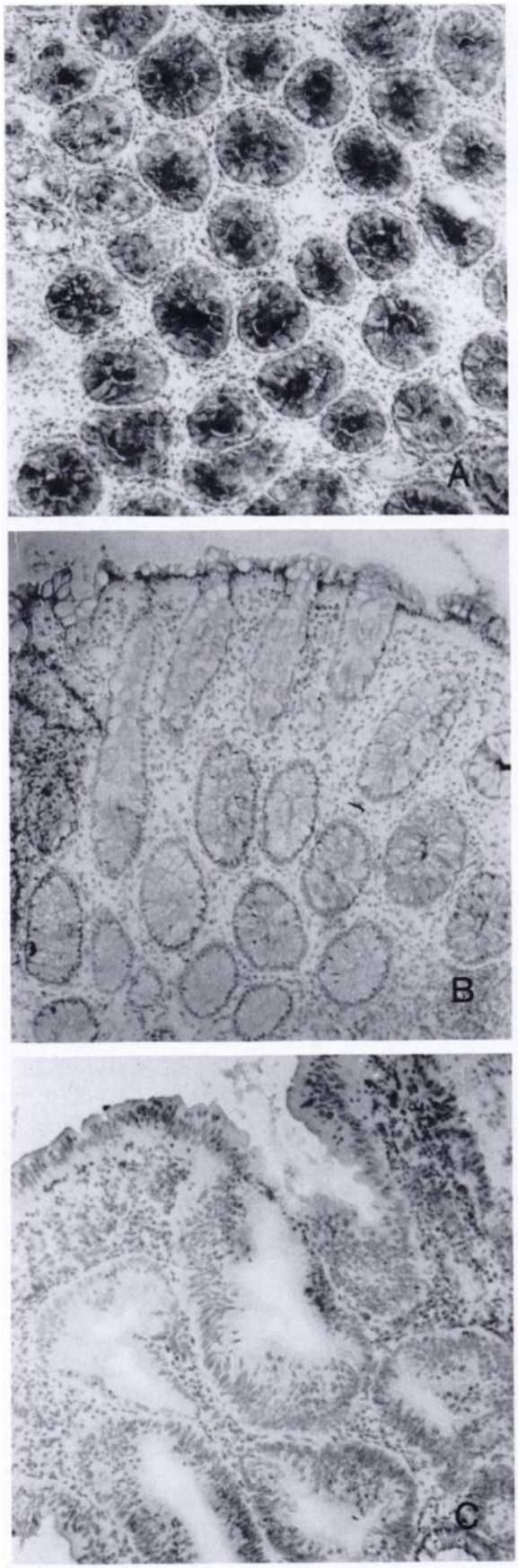

Fig. 2. Immunoperoxidase staining of HLA class I antigens in colonic tissues. Uniform and strongly positive staining can be seen in all colonic crypts in normal subjects (*A*); reduced expression with patchy and heterogeneous staining is evident in both normal (*B*) and adenomatous (*C*) glands from patients with adenoma. Hematoxylin counterstain, × 100.

Table 2 *HLA class I expression in adenomas according to their degree of dysplasia*

Immunoperoxidase scoring in "Materials and Methods." HLA expression correlated to the degree of dysplasia (P 0.001, Kruskal-Wallis test).

Grade of dysplasia	HLA class I expression n (%)					Total
	0+	1+	2+	3+	4+	
Mild	0 (0)	4 (28)	5 (36)	5 (36)	0 (0)	14
Moderate	0 (0)	5 (42)	6 (50)	1 (8)	0 (0)	12
Severe	3 (38)	3 (38)	2 (24)	0 (0)	0 (0)	8
Total	3 (9)	12 (35)	13 (38)	6 (18)	0 (0)	34

HLA class I antigen expression, assessed semiquantitatively by immunoperoxidase, decreased as the neoplastic phenotype developed and correlated with the degree of dysplasia of the adenoma. The heterogeneous pattern of HLA class I antigen expression that was initially observed in colon cancer (8, 13) was also found in adenomas. This perhaps implies that there exists a selective suppression of the HLA class I antigen expression in a gradually increasing subgroup of cells throughout colon tumorigenesis.

In at least two-thirds of the patients who develop adenomas, the mechanism suppressing the normal expression of HLA antigens is operative before histological changes associated with neoplasia become apparent. Furthermore, such suppression appears to be independent of the presence of the adenoma *per se*, because it is manifest following polypectomy. However, the status of HLA class I antigen expression prior to the polypectomy in these patients is unknown. An arbitrary subgrouping of the patients to those with one adenoma and those with two or more adenomas (patients possibly more prone to adenoma) did not yield any significant difference in HLA class I expression between the two groups.

The expression of HLA class I antigens in sporadic adenomas was similar to that previously reported for adenomas from patients with familial adenomatous polyposis (10). This observation contributes further evidence that the phenotypic characteristics of familial adenomatous polyposis adenomas do not differ from sporadic adenomas (14, 15).

The changes that we report here have been detected by immunoperoxidase, using an antibody against the products of three HLA class I loci (A, B, and C). That a composite estimate of the expression of three loci has been made leaves open the possibility that the changes of individual loci may be far greater. Loss of expression of individual class I loci has been reported (6, 7, 16) as well as evidence of noncoordinate expression of HLA loci (17).

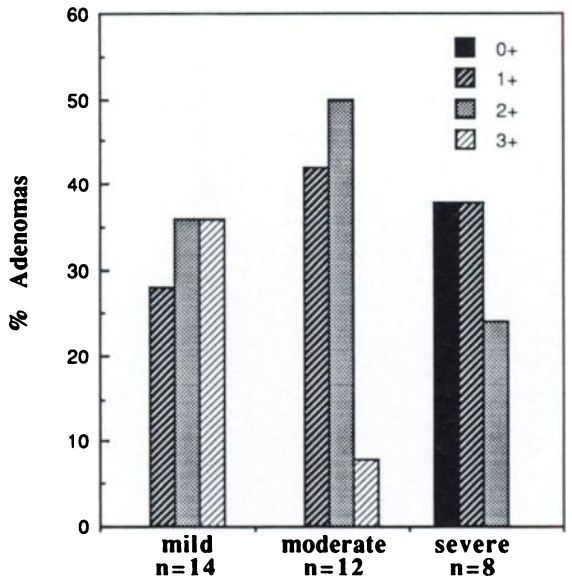

Fig. 3. HLA class I expression in colorectal adenomas according to the grade of dysplasia. Immunoperoxidase as in "Materials and Methods."

Table 3 *HLA class I expression in histologically normal mucosa from patients with one or more adenomas*

Immunoperoxidase scoring as in "Materials and Methods." There was no significant difference in HLA class I antigen expression between the two groups (Mann-Whitney test).

No. of adenomas			
1		2	
Age/sex	HLA	Age/sex	HLA
69M	3+	80M	4+
70M	2+	53M	3+
73M	2+	60M	1+
68M	4+	63M	4+
70M	4+	66M	3+
55M	2+	62M	2+
81M	3+	71M	3+
70M	3+	68M	2+
52M	4+	76M	2+
79M	3+	69M	2+
53M	2+	51M	4+
71M	4+	71M	3+
75M	2+		
67M	3+		
62M	4+		
70M	3+		
64M	3+		
68M	3+		
73M	4+		
73M	4+		
62M	3+		
63M	3+		
69M	3+		
80F	2+		
54M	3+		
77F	3+		
68M	3+		

The reduced expression of HLA antigens that is observed to occur throughout the process of colonic tumorigenesis can be (*a*) an early result of neoplastic transformation, (*b*) a required or contributing event, or (*c*) an independent, unrelated event. The last possibility is rather unlikely, given that the two processes progress in tandem. Our data cannot distinguish between the first two possibilities.

An additional unanswered question is whether the observed changes in HLA expression in histologically normal mucosa may be used as a marker of impending development of colonic neoplasms. Given the high rate of abnormal HLA class I expression in histologically normal colonic mucosa, one could envision attempts to design a screening test to detect people prone to develop colonic tumors.

In conclusion, our data suggest that there is a consistent reduction of HLA class I antigens in colonocytes associated with all premalignant stages of colonic neoplasia and preceding its histological manifestation. These findings raise the possibility that such changes may play a role in the development of colon cancer.

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