Humoral immune response in Japanese acute hepatitis patients with hepatitis C virus infection

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N Yamaguchi, K Tokushige, K Yamauchi, N Hayashi. Humoral immune response in Japanese acute hepatitis patients with hepatitis C virus infection. Can J Gastroenterol 2000;14(7):593-598. The humoral immune response to acute infection by hepatitis C virus (HCV) is not yet perfectly clear in terms of immunoglobulin (Ig) response, diversity of HCV antigen, and the relation with hepatitis severity and antibody response. Serum IgM and IgG anti-HCV levels in patients with HCV and either acute hepatitis (AH) or fulminant hepatitis (FH) were investigated; the diversity of HCV antigen was investigated by RIBA test III. Of 22 AH patients, 12 (54.5%) were positive for IgM anti-HCV, mainly reacting to HCV core protein. The mean interval until the appearance of IgM anti-HCV after onset was 24.1±26.2 days. IgG anti-HCV mainly reacted to both core and NS-3 antigen, appearing 42.6±42.1 days after onset. From a serial study of 15 AH patients, it was considered that in seven AH patients (46.7%), the IgM response would precede the IgG response. In another two AH patients, IgM anti-HCV was not detected during the acute disease phase. Of 48 chronic hepatitis patients with HCV-RNA, 40 patients were positive for IgM anti-HCV. Therefore, IgM anti-HCV was useful for diagnosis in some of the AH patients, but it was difficult to use for distinguishing between acute and chronic infection. All four FH patients with HCV-RNA were positive for both IgM and IgG antibody to HCV at onset. Their antibody titres were higher than those of AH patients. These results suggested that, as in FH due to HBV, FH due to HCV could induce strong and rapid humoral immunity.

Key Words: *Acute hepatitis; Fulminant hepatitis; Immunoglobulin M antihepatitis C virus; RIBA test III*

Réaction immunitaire humorale chez des patients japonais atteints d'hépatite C aiguë

RÉSUMÉ : La réaction immunitaire à médiation humorale consécutive à une infection aiguë par le virus de l'hépatite C (HCV) n'est pas entièrement élucidée sur les plans de la réponse liée aux immunoglobulines (Ig), de la diversité de l'antigène du HCV et du rapport entre l'intensité de l'hépatite et la réaction immunitaire. Les taux d'IgM et d'IgG anti-HCV sériques chez les patients atteints d'une infection au HCV soit aiguë, soit fulminante (HA ou HF) ont fait l'objet de recherches. La diversité de l'antigène du HCV a été analysée par le test RIBA III. Sur 22 patients atteints de HA, 12 (54,5 %) se sont révélés séropositifs à l'égard de l'IgM anti-HCV, réagissant principalement à la protéine nucléocapsidique du HCV. L'intervalle moyen jusqu'à l'apparition de l'IgM anti-HCV après la contamination a été de 24,1 ± 26,2 jours. L'IgG anti-HCV a principalement réagi aux antigènes nucléocapsidique et NS-3, apparaissant 42,6 ± 42,1 jours après le déclenchement. À partir d'une étude sériée de 15 patients souffrant d'HA, on a jugé que chez sept de ces patients (46,7 %), la réponse de l'IgM précéderait la réponse de l'IgG. Chez deux autres patients atteints d'HA, l'IgM anti-HCV n'a pas été décelée durant la phase aiguë de la maladie. Parmi les 48 patients atteints d'hépatite chronique avec ARN du HCV, 40 patients étaient séropositifs à l'égard de l'IgM anti-HCV. Par conséquent, l'IgM anti-HCV s'est révélé utile pour le diagnostic de certains patients atteints d'HA, mais il a été difficile de l'utiliser pour distinguer l'infection aiguë de l'infection chronique. Les quatre patients atteints d'HF avec ARN du HCV étaient positifs à l'égard de l'IgM et de l'IgG dirigés contre le HCV au départ. Leurs titres d'anticorps étaient plus élevés que ceux des patients atteints d'HA. Ces résultats donnent à penser que, comme dans l'HF due au l'HBV, l'HF due au HCV pourrait induire une immunité humorale forte et rapide.

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Detection of immunoglobulin (Ig) M antibody usually precedes that of IgG antibody in viral infection, such as infection by hepatitis A virus (HAV) or hepatitis B virus (HBV). Several studies have indicated that the measurement of serum IgM antihepatitis C virus (HCV) might be useful for the diagnosis of acute infection of HCV (1-3). However, Kikuchi et al (4) reported that IgM response was not observed or was weak in acute HCV infection. We investigated the IgM response with the use of a third generation assay system of anti-HCV, which has good sensitivity, and reassessed the clinical significance of IgM anti-HCV in acute hepatitis (AH).

The single-stranded RNA genome of HCV encodes at least three structural proteins – core, envelope (E) 1 and E2 – and at least six nonstructural (NS) proteins – NS-2, NS-3, NS-4a, NS-4b, NS-5a and NS-5b (5-7). Antibodies against these viral proteins are readily detected with the use of ligands such as synthetic peptides or recombinant proteins (8-13). However, it is still not clear which HCV protein initially induces the antibody response in AH patients. In this study, we examined the response to individual HCV proteins by RIBA test III.

HCV is reported to be one of the causative agents of fulminant hepatitis (FH) (14,15). In FH patients with HBV, antihepatitis B surface (HBs) antibody appeared rapidly (16-18). In FH patients with HBV who were diagnosed by IgM antihepatitis B core (HBc) and HBV-DNA, HBs antigen was sometimes not detected. Therefore, an IgM antibody anti-HBc was considered the most useful serological marker for the diagnosis of FH patients with HBV. Four FH patients were infected with HCV-RNA. We attempted to examine the humoral immune response to HCV in FH patients infected with HCV, and discuss the relationship between the severity of hepatitis and antibody response.

PATIENTS AND METHODS

Patients: Twenty-two AH patients and four FH patients with HCV-RNA were studied. All AH and FH patients were diagnosed both by histological findings and by various serological examinations, including serum HCV-RNA by reverse transcriptase polymerase chain reaction (RT-PCR) (19). These AH patients had no serological markers of HAV, HBV, cytomegalovirus or Epstein-Barr virus, nor did they present with any other obvious causes of hepatitis (alcohol abuse, drug hypersensitivity or autoimmune disease). The pathological findings of all AH patients showed typical features of acute liver injury without chronic changes such as portal fibrosis. As for the infectious source, three had histories of blood transfusion, three had needle accidents, three had operations and two had sexual contact with partners with HCV; 11 had no history of exposure. The AH patients ranged in age from 18 to 78 years and consisted of 10 men and 12 women. All FH patients were diagnosed by the criteria of Takahashi and Shimizu (20). In brief, the patients with normal liver function before the onset of illness, hepatic coma over grade H and elongated prothrombin time (less than 40% of control) were diagnosed as positive.

None of the four FH patients with HCV-RNA had histories of drug exposure or alcohol consumption, and all four were negative for autoantibody. One patient had both HCV and hepatitis G virus (HGV) as detected by RT-PCR (21), and the other three FH patients had no viral markers (IgM anti-HAV, HBs antigen, HBs antibody, hepatitis 'e' antigen, hepatitis 'e' antibody, HBc antibody, IgM-HBc antibody and HBV-DNA by PCR (22), and HGV-RNA by RT-PCR). Their histological changes consisted of massive or submassive necrosis. One survived and three died.

The controls were 11 healthy subjects (with normal liver function and without the above-mentioned viral markers), 10 AH patients (five HBV and five HAV), seven FH patients (negative for HAV, HBV, HCV and HGV markers), 10 chronic hepatitis patients with HBV and 48 chronic hepatitis patients with HCV. All chronic hepatitis patients were diagnosed by histological findings and viral markers (positive for both HCV-RNA and anti-HCV or positive for HBs antigen).

Antibody assay: IgM and IgG anti-HCV were measured by third generation HCV antibody assay kit (HCV EIA 3.0, Abbott Laboratories, USA), in which a peroxidase-conjugated goat affinity-purified antibody to human IgM or IgG (Cappel, USA) was used as a second antibody. This assay enabled the detection of antibodies to HC-34, HC-43, c100-3 and NS-5. Serum samples were diluted 1:40 (IgM and IgG) in 200 µL of specimen diluent. In these dilutions, beads with previous HCV antigen adhering to their surface were incubated for 2 h at 40°C while being shaken. After three washings, the beads were loaded with 200 µL of peroxidase-conjugated goat affinity-purified antibody to human IgM or IgG and incubated for 30 min at 40°C while being shaken. The beads were washed three times and incubated for 30 min at room temperature with 300 µL of peroxidase substrate (3 mg/mL *O*-phenylenedamine, 0.02% [volume/volume] hydrogen peroxide). The reaction was stopped with 300 µL of 2 mol/L sulphuric acid, and the colour was read with a reader at a wavelength of 492 nm with a reference beam at 570 nm. The maximum optical density titre of IgG was 2.0 and that of IgM was 3.0. Three control sera from healthy individuals with normal liver function were included in each assay. All samples were assayed in duplicate.

RIBA test III: The sera of AH patients were analyzed for IgM and IgG anti-HCV to individual HCV proteins by HCV RIBA test III (Ortho Diagnostics Systems, USA). The assay kit contained the following recombinant antigens: c22p (core), c33c (NS-3), c100p (NS-4), NS-5, superoxide dismutase and anti-IgG antibody. Analysis was carried out according to the instructions of the manufacturer and Buffet et al (23). In brief, 20 µL (IgG) and 40 µL (IgM) of sera with 1 mL of dilution buffer were incubated with a strip adhering these HCV antigens and shaken for 4 h. After three washes with phosphate-buffered saline, a second antibody of the same antibody described in the antibody assay was added and incubated for 10 min. After three washes, the substrate solution was added and the density of the band was determined.

TABLE 1
Clinical and laboratory data of acute hepatitis patients with hepatitis C virus (HCV) infection

Patient	Age (years)/sex	Source of infection	AST peak (IU/L)	Interferon treatment	HCV-RNA of endpoint
A-1	21/F	Unknown	714	–	Sustained
A-2	26/F	Needle accident	1010	+	Cleared
A-3	24/F	Needle accident	751	+	Cleared
A-4	61/M	Operation	1449	+	Sustained
A-5	29/M	Unknown	1354	+	Cleared
A-6	41/F	Unknown	1826	–	Sustained
A-7	24/F	Needle accident	1068	+	Cleared
B-1	78/F	Unknown	1173	–	Cleared
B-2	51/M	Unknown	2185	–	Sustained
B-3	32/M	Unknown	644	+	Cleared
B-4	47/M	Unknown	2952	–	Sustained
C-1	61/M	Operation	319	–	Sustained
C-2	41/M	Unknown	3690	+	Cleared
D-1	72/M	Transfusion	344	+	Sustained
D-2	26/F	Sexual contact	1050	+	Cleared
E-1	18/F	Unknown	1072	–	Cleared
E-2	36/F	Transfusion	1538	–	Sustained
E-3	28/F	Sexual contact	1902	+	Cleared
E-4	78/M	Transfusion	415	–	Sustained
E-5	20/M	Unknown	2731	+	Sustained
E-6	56/F	Operation	351	–	Sustained
E-7	26/F	Unknown	204	+	Sustained

AST Aspartate aminotransferase; F Female; M Male

Statistical analysis: Statistical analysis was performed by unpaired *t* test. $P<0.05$ was considered significant.

RESULTS

Prevalence of IgM to HCV in acute liver diseases: Twenty-two AH patients and four FH patients with HCV-RNA were analyzed (Tables 1,2). Twelve of the 22 AH patients received interferon therapy. Ten AH patients were cleared of HCV-RNA – eight by IFN therapy and two by the natural course. The others became chronically infected.

The IgM response in the various liver diseases was investigated (Figure 1). The cutoff titer of IgM anti-HCV (0.531) was determined by adding three standard deviations to the mean titre of non-HCV hepatitis patients and healthy controls. IgM antibody to HCV was detected in 12 (54.5%) of the 22 AH patients with HCV, all four (100%) FH patients with HCV and 40 (83.3%) of 48 CH patients with HCV (Figure 1); however, there was no correlation among IgM response and resolution, serum alanine aminotransferase and serum bilirubin of AH. In comparison, this antibody was not detected in 11 healthy subjects, all 10 AH patients with HAV or HBV, seven FH patients (non-A, non-B, non-C) and 10 CH patients with HBV.

Titre (mean ± SD) of IgM anti-HCV was highest in FH patients with HCV (2.072±1.094), followed by CH patients with HCV (1.816±1.108) and then AH patients with HCV (0.943±0.885). Table 2 shows the data of IgM and IgG anti-HCV in FH patients with HCV-RNA. In all FH patients, both antibodies were already detected at onset. These results suggest that fulminant hepatic failure with HCV might induce rapid and strong humoral immunity, as occurs in FH induced by HBV. In contrast to CH patients with HBV, IgM anti-HCV was detected in the majority of CH patients with HCV.

TABLE 2
Immunoglobulin (Ig) M and IgG antibodies in fulminant hepatitis patients with hepatitis C virus (HCV)-RNA

Patient	Age/sex	IgM anti-HCV	IgG anti-HCV	HCV-RNA (PCR)	HCV-RNA (probe)
1	40/F	>3.0	>2.0	+	<0.24 Meq/mL
2	31/M	>3.0	>2.0	+ (HGV+)	Not done
3	56/F	0.871	>2.0	+	<0.24 Meq/mL
4	47/M	1.419	>2.0	+	Not done

HGV Hepatitis G virus; PCR Polymerase chain reaction

Serial study of AH patients: Serial samples from 15 AH patients with HCV-RNA were assayed to establish the pattern of appearance of IgM and IgG anti-HCV (Figure 2). In the initial samples, 10 (66.7%) were positive for IgM anti-HCV and nine (60%) for IgG anti-HCV. IgG anti-HCV eventually became positive in all AH patients. IgM appeared be-

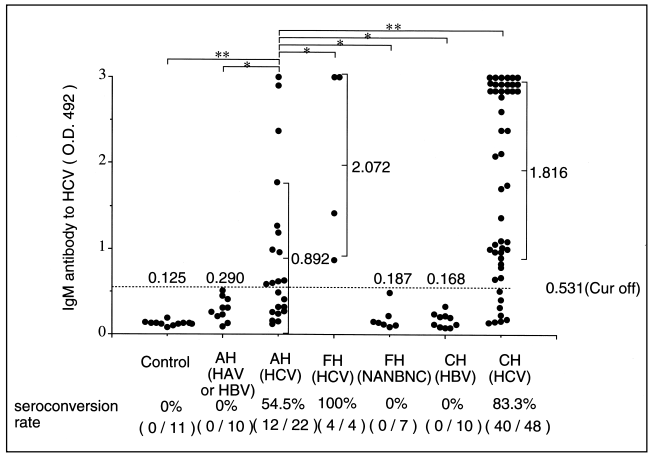

Figure 1) *The titre of immunoglobulin (Ig) M antihepatitis C virus (HCV) in various diseases. The cutoff titre of IgM anti-HCV (0.531) was determined by adding three standard deviations to the mean titres of non-C hepatitis patients and healthy controls. IgM antibody to HCV was detected in 12 (54.5%) of the 22 acute hepatitis (AH) patients with HCV, all four (100%) fulminant hepatitis (FH) patients with HCV and 40 (83.3%) of 48 chronic hepatitis (CH) patients with HCV. Mean IgM antibody titres of each group are shown. In comparisons of patients with AH due to HCV, *P<0.05; **P<0.01. HAV Hepatitis A virus; HBV Hepatitis B virus; NANBNC Non-A, non-B, non-C; OD Optical density*

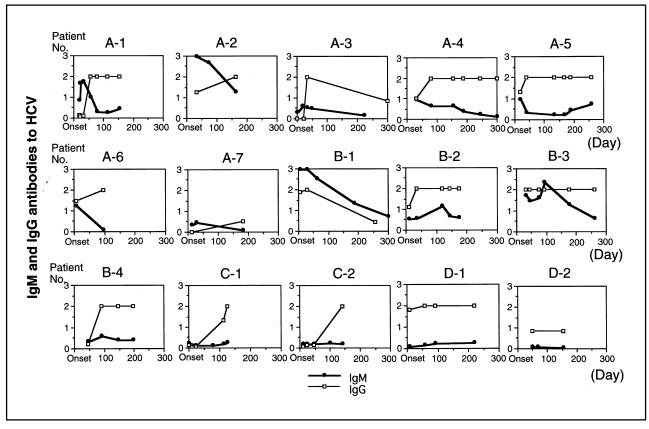

Figure 2) *Serial study of immunoglobulin (Ig) G and IgM antihepatitis C virus (HCV) in 15 patients with acute hepatitis*

tween zero and 91 days after the onset of symptoms (mean ± SD 24.1±26.2 days), and IgG anti-HCV appeared between four and 140 days (42.5±42.1 days) after the onset of symptoms. In patients A-1, A-2, A-3 and A-7, IgM antibody response preceded that of IgG, and an Ig class switch from IgM to IgG against HCV was observed. A class switch was not detected in patients A-4, A-5 and A-6, but from the pattern of two antibodies, it was presumed that the IgM response would have preceded the IgG response to HCV. Of 15 AH patients, seven AH patients (46.7%) were considered to have undergone an Ig class switch. In patients B-1, B-2, B-3 and B-4, IgM became positive later or simultaneously with the IgG response. IgM anti-HCV was not detected in patients C-1 and C-2 during the acute disease phase, which occurred even before IgG anti-HCV was detected. In patients

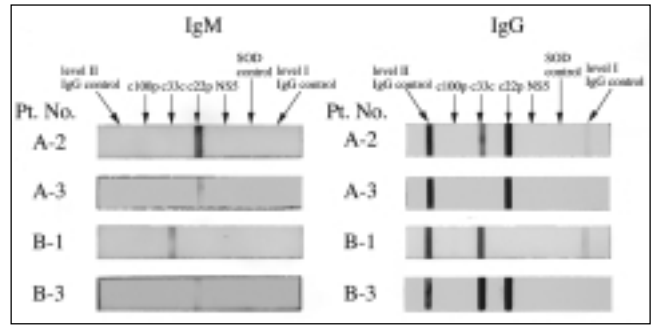

Figure 3) *Immunoglobulin (Ig) M and IgG responses to individual hepatitis C virus (HCV) antigens by HCV RIBA test III in representative acute hepatitis cases. A common IgG band in the IgM assay was not detected, suggesting that the second antibody in the assay system showed no crossreactivity with IgG. Pt. No. Patient number; SOD Superoxide dismutase*

TABLE 3
Reactivity for individual hepatitis C virus (HCV) protein of immunoglobulin (Ig) M and IgG antibodies in patients with acute hepatitis patients with HCV

	C22p (core)	C33c (NS-3)	core+NS-3	C100p (NS-4)	NS-5
IgM	9/11 (81.8%)	1/11 (9.1%)	0/11 (0%)	1/11 (9.1%)	0/11 (0%)
IgG	4/18 (22.2%)	6/18 (33.3%)	8/18 (44.4%)	0/18 (0%)	0/18 (0%)

NS Nonstructural protein

D-1 and D-2, the IgG response had already reached a maximum level when the first samples were measured. Thus, in these group D cases, it might have been too late to measure the IgM response of AH, although the clinical, serological and pathological findings of these two patients were indicative of AH.

Antigen diversity of antibody in AH patients: AH patients were analyzed for reactivity of IgM and IgG antibodies to individual HCV protein by RIBA test III. Figure 3 shows representative cases. A common IgG band for the controls was not found on examination of IgM antibody, suggesting that the second antibody to human IgM had no crossreactivity with IgG antibody. In nine of 11 AH patients, IgM antibodies mainly reacted to c22p (HCV core) antigen. In the other two, one was to c33e (NS-3) antigen and the other to c100p (NS-4) antigen (Table 3). Regarding IgG anti-HCV, in eight of 18 AH patients, IgG antibodies reacted to both core and NS-3 antigen, six to NS-3 and four to core antigen.

DISCUSSION

We reassessed the clinical features of IgM anti-HCV in AH patients with HCV-RNA. In this study, a third generation HCV antibody assay system (Abbott) was used. Previous studies used measurements by IgM anti-HCV core or C100-3 protein (14). IgM antibody in our system was recognized with core, NS-3, NS-4 and NS-5 proteins. The sensitivity of our system was considered to be higher that that found in other

studies. As shown in Table 2, IgM antibodies of two AH patients recognized NS-3 or NS-4.

The question of the possibility of false positive must still be answered. If the patients have rheumatoid factor and IgM antibody reacts to an IgG, an IgG control band must be observed in the IgM RIBA III test. An IgG control band was never detected throughout the IgM RIBA III test. In addition, we measured IgM anti-HCV in 11 healthy controls and 27 non-HCV hepatitis patients, which were all negative. Therefore, we believe that there was no false positive. As for the possibility of false negative, in AH patients with HCV, the amount of IgG anti-HCV was small compared with that of CH patients with HCV. Especially in AH cases in whom the IgM response preceded the IgG response, IgG anti-HCV antibody did not compete with IgM anti-HCV antibody even though IgG was not removed from serum.

In our AH patients with HCV-RNA, IgM anti-HCV was detected in 55%. Sato et al (3) reported almost the same results, with 11 of 27 AH patients (40.7%) being AH-positive for IgM anti-HCV. In our study, 46.7% of AH patients indicated that the IgM response would precede the IgG response, similar to observation of acute infection by HAV or HBV. However, in two AH cases, as shown in Figure 2, we did not detect IgM anti-HCV during the acute disease period. Two possibilities for this negative IgM response were considered. One was related to the quantity of HCV-RNA. Many studies have indicated that low levels of circulating HCV-RNA, and thus viral particles (10^4 to 10^8/mL), are present in serum during HCV infection (24,25) compared with HBV-DNA. Therefore, HCV might not induce the initial humoral immunity effectively. The other possibility concerned the time of the appearance of IgM anti-HCV because it might have appeared much earlier.

IgM and IgG anti-HCV were more frequently detected and the titre was higher in FH patients with HCV than in patients with ordinary AH. Akbar et al (26) reported that, of eight patients with FH and subacute hepatitis, three (37.5%) were positive for IgM anti-C100-3, a difference possibly related to the use of a different antigen in the assay system. In patients with FH due to HBV, anti-HBs antibody also appeared earlier than in patients with ordinary AH due to HBV (16-18). We speculated that severe liver damage can induce strong and rapid humoral immunity through several cytokines. We reported that the serum level of interleukin-6, which could change B cells to antibody-producing cells, was markedly high in FH patients (27). The present results support our hypothesis. The possibility was also raised that HCV infects patients chronically and that some other virus or factor induces acute hepatic failure. However, the major hepatic pathological finding in all four FH patients was massive necrosis without chronic change, such as portal fibrosis. One patient was infected with HCV and HGV. The other three FH patients were negative for other virus markers except HCV, were negative for autoantibody, and had no history of drug or alcohol abuse. From the clinical and serological data and histological findings, we believe that acute hepatic failure was induced by HCV in these four patients. In fact, HCV has already been reported to induce fulminant hepatic failure (14,15).

As for the diversity of HCV antigens, our study indicated that IgM antibodies were induced by HCV core antigen and IgG antibodies by HCV core and NS-3 antigens. Our examination of HCV antigen diversity for IgM antibody appears to be the first reported. Regarding the IgG response, Chen et al (28) reported that the HCV core antigen was more immunogenic than other viral antigens in AH patients. They stated that the reason for the difference in immune response to HCV core antigen compared with other viral antigens was not clear, but the core protein was considered to be expressed at higher levels than other proteins. Animal studies of the immunogenicity of HCV core (29), NS-3 (30) and NS-4a (31) antigens have shown that recombinant versions of these HCV proteins delivered as adjuvants elicit respectable levels of antibody. However, the immune responses were different between natural infection and induction with recombinant proteins. In any case, HCV core antigen could be expected to at first induce humoral immune response, as does HBV core antigen.

IgM antibody is usually detected in the acute phase of viral infection and then disappears. However, IgM antibody to HCV is detected more frequently in chronic hepatitis. Yuki et al (32) and Brillanti et al (33) reported that IgM antibody to HCV was correlated with viral replication and hepatitis activity and was a good marker of antiviral therapy. We found the IgM antibody to HCV to be a good parameter for IFN therapy, especially genotype 1b (data not shown). The mechanism of the appearance of IgM antibody in chronic HCV infection was unclear. As one possibility, a relation between B cell lymphoma and HCV chronic infection has been reported (34,35). In this regard, chronic infection with HCV might stimulate B cell lymphocytes and secrete IgM antibody. In any event, it was difficult to distinguish between acute infection and chronic infection solely by the IgM antibody to HCV.

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