Hepatitis C and Hepatitis B in the Etiology of Hepatocellular Carcinoma in the Japanese Population

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ABSTRACT

We conducted case-control studies of hepatocellular carcinoma (HCC) and liver cirrhosis (LC) in relation to hepatitis C virus (HCV) and hepatitis B virus infection, involving 91 patients with HCC, 75 patients with LC who had no evidence of HCV, and 410 control subjects from the Japanese population. Serum antibody to HCV (anti-HCV) was detected by both enzyme-linked immunosorbent assay and recombinant immunoblot assay in 31, 51, and 3% of HCC, LC, and controls, respectively, whereas the corresponding prevalence of serum hepatitis B surface antigen (HBsAg) was 21, 11, and 2%, respectively. The relative risks (and 95% confidence intervals) for the presence of serum anti-HCV were estimated as 52.3 (23.9–114.3) for HCC and 64.4 (27.4–151.4) for LC. These values exceeded the relative risk of HCC (15.3) and that of LC (6.1) for positive serum HBsAg. Among male patients with HCC or LC, anti-HCV rates were very high in blood recipients (about 70%), heavy drinkers (46–62%), and those who had no identifiable risk factors (65–75%), indicating possible transmission of HCV via routes other than transfusion. No significant difference in anti-HCV status was observed between the HCC and LC groups. It was notable that anti-HCV was much less prevalent among HBsAg-positive patients with HCC or LC than among HBsAg-negative ones. There was a slight to moderate increase in HCC or LC risk among blood recipients and heavy drinkers after adjustment for anti-HCV status. These results indicate that, in Japan, the possible role of HCV infection in the etiology of HCC and LC is extremely large and seems to be more important than chronic hepatitis B virus infection.

INTRODUCTION

To date, the most important risk factor for HCC has been chronic HBV infection (1–3). In fact, a great majority of HCC patients in the regions endemic for the disease such as sub-Saharan Africa and southeast Asia seem to be attributed to chronic HBV infection (4, 5). Japan is also among the relatively high risk areas (6). However, in this country, most HCC patients (about 75%) in recent years have been negative for serum HBsAg (7), a marker which, when testing positive, usually indicates a chronic HBV carrier state. Although Bréchot et al. (8) reported that HBV DNA was frequently detected in liver tissue from HBsAg-negative patients with HCC and thus HBV may play a role in those patients, such evidence has not been confirmed in studies using the same approach in Japan (9, 10).

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Hence, risk factors other than chronic HBV infection, especially infection with unidentified non-A, non-B hepatitis virus or viruses, have been suspected to be important in the etiology of HCC in Japan (10–12).

In 1989, Choo et al. (13) reported the discovery of complementary DNA presumed to be derived from a blood-borne non-A, non-B hepatitis virus. They designated the virus as HCV, and now an assay for anti-HCV has become available (14). We have conducted case-control studies of HCC and LC especially focusing on the role of HCV and HBV infection in Fukuoka prefecture, where the risk for HCC is one of the highest in Japan (15). In this area, liver cancer is the second and third leading cause of cancer deaths in men and women, respectively (crude death rate per 100,000 of liver cancer in 1987: men, 47.8; women, 15.9).

MATERIALS AND METHODS

Study Subjects. We initiated a case-control study of HCC in relation to various possible risk factors in 1985. In addition to determining serum HBV markers, a detailed interview survey on past histories of blood transfusion, alcohol consumption, cigarette smoking, etc., was conducted in the study. The methods and part of the results obtained have been reported previously (12). In brief, patients with HCC were eligible if the following criteria were met: (a) diagnosed as HCC initially within 1 year prior to identification; (b) ages 40 to 69 years (patients ages 70 years or over as well as those under 40 years were excluded since the former may lack reliability in their responses about their past histories and the latter presented a practical difficulty in obtaining adequate controls with respect to age); (c) residents in Fukuoka or Saga Prefecture (neighboring Fukuoka prefecture) of Japanese nationality. Patients with HCC were identified among those who were admitted to the First and Third Departments of Internal Medicine and the Second Department of Surgery of Kyushu University Hospital from December 1985 to June 1989 by contact with physicians as well as by a weekly review of the list of hospitalized patients and the admission reports. Finally, a total of 204 Japanese patients with HCC who met the above criteria were investigated. Among the 204 patients, the sera from 91 patients were stored and available for this study. These 91 patients did not significantly differ from the remaining 113 patients without their stored sera in the prevalence of serum HBsAg and histories of blood transfusion and alcohol consumption. Eighty-nine of the 91 patients were residents of Fukuoka prefecture, while 2 were from Saga prefecture. The major diagnostic methods for HCC in the 91 patients were: 30 histologically confirmed; 58 based on angiographic findings (16); 3 based on the findings of ultrasonography and computed tomography, elevated serum a-fetoprotein, etc. Preexisting liver diseases among the 91 patients were: 76 with liver cirrhosis (25 histologically confirmed; 1 confirmed by laparoscopy; 50 clinically established); 2 with chronic active hepatitis; 3 with chronic persistent hepatitis; 4 with chronic hepatitis without available histology; 1 with no diagnostic abnormality; 5 unknown. Of the 91 patients: 19 (21%) were serum HBsAg positive; 22 (24%) had positive history of blood transfusion due to causes other than liver diseases; 27 (30%) had positive history of heavy drinking (80...
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ml or more of ethanol per day for at least 10 years); 34 (37%) had no identifiable risk factors; 7 patients had double factors (2 with positive HBsAg and transfusion; 1 with positive HBsAg and heavy drinking; 4 with positive transfusion and heavy drinking); and 2 patients had triple factors.

In parallel with the case-control study, 100 Japanese patients with LC were also investigated at the same departments during the period from December 1985 through December 1987 according to the same factors. Patients with liver cirrhosis who met the above criteria of a and c and who had no evidence of HCC by available diagnostic methods such as ultrasonography, computed tomography, and serum α-fetoprotein at the time of identification were regarded as eligible. Special types of LC such as primary and secondary biliary cirrhosis and that due to autoimmune hepatitis, parasitosis, congestive heart failure, or metabolic disorders were excluded because they appeared to differ substantially from the most common type of LC observed in Japan. Because of a possible bias in hospitalized patients with LC with respect to relevant factors, most (67%) of the patients were selected from among the outpatients. Among the 100 patients, the sera from 75 patients were available for this study. Seventy-two of these 75 patients were residents of Fukuoka prefecture. The diagnoses of LC for the 75 patients were based on the following methods: 24 histologically confirmed; 14 confirmed by laparoscopy; 37 based on evident clinical signs and the findings in imaging methods such as ultrasonography (17) and laboratory data. Of the 75 patients: 8 (11%) were serum HBsAg positive; 21 (28%) had positive history of blood transfusion due to causes other than liver diseases; 14 (19%) had positive history of heavy drinking; 41 (55%) had no identifiable risk factors; and 9 patients had double factors (3 with positive HBsAg and transfusion, and 6 with positive transfusion and heavy drinking).

Controls comprised 410 persons ages 40 to 69 years who were residents of Fukuoka City and who visited a public health center located near Kyushu University Hospital between January 1986 and July 1989 to undergo health examinations. The controls were selected so that their distribution by sex and age was as similar as possible to that of patients with HCC or LC. Those who suffered from chronic liver diseases such as chronic hepatitis and liver cirrhosis, or from whom blood specimens could not be obtained, were excluded. Sera from all the controls were stored and utilized for this study. The distribution of study subjects by demographic characteristics is shown in Table 1. As for occupation, subjects were classified according to the jobs in which they had been engaged longest. No patients with HCC or LC had experienced occupational exposure to special chemical substances. There were some differences among study groups in the following points: the age between male HCCs and male controls (P < 0.05 by Wilcoxon test); occupations between male HCCs and male controls; years of education between female HCCs and female controls and between female LCs and female controls (P < 0.01 by Wilcoxon test). These factors were accommodated by the statistical procedures in estimating the relative risks for relevant factors because of their possible confounding effects.

Interviews. Concerning alcohol consumption, history of blood transfusion, and other relevant factors in addition to demographic characteristics, all study subjects were interviewed in person by three trained interviewers. During the first 2.5 years, one person interviewed both patients and controls, and over the next 2 months another interviewer conducted interviews while taking turns with the first interviewer due to logistic reasons, and the last interviewer conducted all interviews for the rest of the study period. Most of the subjects were interviewed by the first interviewer. To obtain accurate information, all interviews were tape recorded and checked against questionnaires by both the interviewers and one of the authors (K. Tanaka). Subjects were interviewed in a private room in the hospital wards or in the outpatient clinic or at the health center.

Laboratory Tests. Information on serum HBV markers was obtained from medical records for patients; all patients were tested for serum HBsAg by radioimmunoassay or reversed passive hemagglutination; the status of antibody to hepatitis B core antigen or antibody to HBsAg was not determined in most of the patients. For the controls, sera were tested for HBsAg by the reversed passive hemagglutination method. Sera from 91 patients with HCC, 75 patients with LC, and 410 controls were kept frozen at −70°C in the same deep freezer until tested for anti-HCV. The Ortho-HCV ELISA (Ortho Diagnostic Systems, Raritan, NJ) was used to detect anti-HCV. This indirect assay uses a recombinant HCV antigen polypeptide (C100-3, described in detail by Choo et al. (13). Anti-HCV in the serum binds solid-phase HCV antigen. The specifically captured antibody is revealed with an antibody–enzyme conjugate composed of a murine monoclonal antibody against human IgG and the enzyme, conjugated with horseradish peroxidase. The test was performed according to the manufacturer’s instructions. The results were read at 492 nm by an ELISA plate reader with an upper limit of 3.0 absorbance units. The cutoff in the assay, which was calculated as the mean absorbance of the three negative manufacturer’s controls plus 0.400 absorbance unit, ranged from 0.438 to 0.455 (mean, 0.444). The positive controls supplied with the kits gave a reading from 1.662 to 2.252 (mean, 1.956). Positive results were confirmed in all subjects by retesting in duplicate or triplicate, as prescribed by the manufacturer. Furthermore, the ELISA-positive sera were reexamined by the Chiron HCV RIBA (Chiron Corp., Emeryville, CA), distributed by Ortho Diagnostic Systems. This assay includes the three recombinant antigens, i.e., SOD-HCV fusion polypeptides 5-1-1 (synthesized in Escherichia coli), C100-3 (synthesized in yeast) and SOD alone (synthesized in yeast), applied to a nitrocellulose strip. High and low levels of human IgG are also incorporated onto each strip as positive controls. Twenty ml of the serum from each study subject were incubated for 4 h at room temperature with the antigens on the strip, and then the strip was reacted with goat anti-human IgG labeled with horseradish peroxidase. The test was done according to the manufacturer’s instructions. The results are read by comparing the colors of the antigen bands with those of the positive controls: negative, no visible band; ±, visible but intensity less than low level control; 1+, intensity equal to low level control; 2+, intensity greater than low level but less than high level control; 3+, intensity equal to high level control; 4+ intensity greater than high level control. Results of 1+ or greater were regarded as positive for each antigen. According to the manufacturer’s instructions, samples positive for both 5-1-1 and C100-3 are “reactive”; those positive for either 5-1-1 or C100-3 (or for SOD and one or both of the HCV antigens) are “indeterminate”; and samples positive for SOD and/or none of the HCV antigens are “nonreactive.”

Statistical Analysis. χ² and Fisher’s exact tests were used for the unadjusted comparison of study groups. A Mantel-Haenszel test (18) was performed for a stratified analysis with a few factors controlled. The RRs (more correctly odds ratios) of HCC or LC for selected risk factors were estimated by modeling the data through unconditional logistic regression for controlling possible confounding factors such as sex, age, etc., using the SAS statistical package (19, 20). In the model, data on either patients with HCC or LC as well as controls were included, and the RRs of HCC or LC were calculated. The natural
antilogarithm of regression coefficients of indicator variables is interpreted as the RR for HCC or LC, and the 95% CI was similarly obtained from the regression coefficient and its standard error. All reported \( P \) values are two-tailed.

RESULTS

Sixty-two (68%) of 91 patients with HCC, 48 (64%) of 75 patients with LC, and 28 (7%) of 410 controls were found to be positive for anti-HCV in the ELISA. In our serum samples, positive rates in the ELISA were not associated with storage duration of the sera or with the departments (surgery or internal medicine) to which the patients were admitted (data not shown). Table 2 shows the distribution of the ELISA-positive subjects according to absorbance values in the ELISA and the results from the RIBA. Among the ELISA-positive subjects, 46 (74%) of 62 patients with HCC, 38 (79%) of 48 patients with LC, and 12 (43%) of 28 controls were positive for both C100-3 and 5-1-1, i.e., “reactive” in the RIBA. Reactive rates in the RIBA were closely correlated with absorbance values in the ELISA; among the subjects with absorbance values less than 2.0, only 1 control was RIBA reactive, whereas, among those with 2.0 or greater absorbance values, the reactive rates were 85, 88, and 58% for HCC, LC, and controls, respectively. No visible bands for SOD in the RIBA were detected through our examinations.

Table 3 presents the prevalence of anti-HCV among study subjects by results from ELISA and RIBA and by sex. The prevalence of RIBA-reactive results among patients with HCC, patients with LC, and the controls were 58.9%, 58.3%, and 3.8%, respectively, for males and 16.7%, 37.0%, and 0.8%, respectively, for females. The difference in these figures was statistically highly significant both for males \( (\chi^2 = 161.6, \text{d.f.} = 2, P < 0.001) \) and for females \( (\chi^2 = 38.6, \text{d.f.} = 2, P < 0.001) \), and that was the case when stratified by age group (data not shown). The difference observed was due to a higher prevalence of anti-HCV among patients with HCC or LC than among controls; anti-HCV status among patients with HCC was not significantly different from that among patients with LC. The RIBA-reactive rates were significantly higher in males than in females after being adjusted for age and study group (Mantel-Haenszel \( \chi^2 = 14.1, P < 0.001 \)).

Table 4 shows the distribution and the prevalence of RIBA anti-HCV by selected risk factors for HCC and LC. Only RIBA-reactive results were included in Table 4. Among those subjects positive for serum HBsAg, the prevalence of anti-HCV ranged from 0 to 20%, which was lower than that shown in Table 3. Among subjects with positive history of blood transfusion, 8 patients with HCC and 17 patients with LC experienced blood transfusions related to liver diseases, e.g., due to bleeding from esophageal varices, etc., within the past decade or so. Since their histories of blood transfusion appeared not to be causative events but to be the results of preexisting liver diseases such as cirrhosis, they were not included in the blood recipients hereafter. Among male blood recipients, about 70% of patients with HCC or LC were anti-HCV reactive in contrast to 13% of controls \( (P < 0.01) \). Among females, such an association was less clear, but the number of subjects was limited. One notable finding was the high prevalence (46% to 75%) of anti-HCV among male patients with HCC or LC who had been heavy drinkers (80 ml or more of ethanol per day for at least 10 years) or who had no identifiable risk factors. Such values were comparable to those detected in blood recipients with HCC or LC. The anti-HCV status was significantly different among the study groups with positive history of heavy drinking or with no identifiable risk factors. Those differences arose from the higher prevalence of anti-HCV among patients with HCC or LC than controls; no significant difference of anti-HCV status was observed, however, between the HCC and LC groups.

Table 5 displays the distribution of study subjects by HBsAg status and RIBA anti-HCV status. Among patients with HCC or LC, there was a highly negative correlation between HBsAg status and anti-HCV status \( (P < 0.05) \); patients with HCC or LC positive for HBsAg tended to be much less reactive for RIBA anti-HCV than those negative for HBsAg. Such a relationship was not significant in the controls, but no controls positive for HBsAg were RIBA reactive. Of the 2 patients with HBsAg-positive and RIBA-reactive results, 1 patient with HCC had received a transfusion.

The RRs of HCC and LC for selected risk factors were estimated with and without adjustment for RIBA anti-HCV status (Table 6). All RRs were adjusted for sex, age, occupation, and years of education regardless of anti-HCV status. The RRs (and 95% CIs) of HCC and LC for individuals with RIBA anti-HCV reactive results compared to those with ELISA-negative or RIBA-nonreactive results were 52.3 (23.9–114.3) and 64.4 (27.4–151.4), respectively. Those values exceeded the RRs for chronic HBV infection as indicated by positive serum HBsAg (15.3 for HCC; 6.1 for LC). It was difficult to adjust the anti-HCV status in estimating the RR for chronic HBV infection because of the highly negative correlation between them as shown in Table 5. Among blood recipients, the RRs of HCC and LC were 3.8 and 4.7, respectively, without adjustment for anti-HCV. After an adjustment was made for anti-HCV, those figures decreased to 2.0 and 2.7, respectively. There was a moderate to slight risk increase among heavy drinkers (RR for HCC, 2.2; RR for LC, 1.4). This association did not change significantly after being controlled for anti-HCV.

DISCUSSION

This controlled study revealed an extremely strong relationship between anti-HCV status and HCC or LC in the Japanese population. The RR of HCC or LC for the presence of anti-HCV exceeded that for positive HBsAg. Yu et al. (21) reported that the RR of HCC for positive anti-HCV in the ELISA was 10.5, but our estimate (52.3) was considerably higher than their figure. The same strong association has been demonstrated in Spain (22), Italy (23), and South Africa (24).

Johnson and Williams (25) recently expressed doubts as to the reliability of anti-HCV results in the ELISA among patients with HCC. They suspected that the higher prevalence of ELISA anti-HCV among those patients could only be false positive results due to hyperglobulinemia which is often seen in HCC.
patients who have coexistent chronic liver diseases. Therefore, we examined the correlation between absorbance values in the ELISA and serum globulin concentration among patients with HCC (Fig. 1). Only a weak correlation was found (Kendall rank correlation coefficient, 0.23; \( P = 0.006 \)), and it is unlikely, according to our data, that the higher prevalence of anti-HCV is simply due to hyperglobulinemia. Similar results were obtained for patients with LC (Kendall rank correlation coefficient, 0.23; \( P = 0.006 \)). Also, we retested the ELISA-positive sera by the RIBA, in which reactive results have been shown to be closely associated with the infectivity of HCV (26) or detection of HCV RNA by polymerase chain reaction (27). In the present study, more than 70% of the ELISA-positive sera from patients with LC were RIBA reactive. These results highly support the significant involvement of HCV infection in the etiology of HCC and LC in the Japanese population.

In this study, anti-HCV was more prevalent in males than in females. Although this phenomenon has not been observed in other studies in foreign countries (22-24), the same tendency of anti-HCV with respect to sex has been found among blood donors in Fukuoka prefecture (see later). Also noted was the high prevalence (46-75%) of anti-HCV in heavy drinkers and individuals with no identifiable risk factors as well as blood recipients among male patients with HCC or LC. Although HCV was found as a transfusion-related agent at first, there may be other routes of transmission. Furthermore, HCV may play a role in patients with LC which is presumably due to heavy drinking and patients with HCC after suffering from so-called "alcoholic" cirrhosis. There was no significant difference in anti-HCV status between the HCC and LC groups.

A highly negative correlation between anti-HCV status and HBsAg status was observed among patients with HCC or LC in the present study; the prevalence of anti-HCV was much lower among HBsAg-positive patients with HCC or LC than among HBsAg-negative ones. Among the controls such a relation was not clear, but the prevalence of HBsAg and anti-HCV in the control group might have been too low to detect such an association. Evidence for a negative correlation between anti-HCV status and HBsAg status has not been found in reports from other countries (22-24), but in Japan similar results have been obtained (28-31). The reason of this discrepancy cannot be explained at present, and further investigations are needed.

Yu et al. (21) found a synergistic effect of past or current HBV infection and HCV infection on HCC risk, but we could not evaluate such an effect.

Histories of blood transfusion and heavy drinking were also risk factors for HCC and LC in this study. After an adjustment was made for anti-HCV, the RR for blood recipients was reduced substantially but remained elevated (about 2-3-fold as compared with nonrecipients). Thus, the assays available at present may be undetectable for part of HCV infection, or there may be other transfusion-related agents. By contrast, the RR for positive history of heavy drinking changed little after being controlled for anti-HCV. Taking the overall high prevalence of anti-HCV among patients with HCC or LC into consideration, heavy alcohol consumption may promote hepatocarcinogenesis among those infected with HCV or other viruses.

Table 7 shows the prevalence of serum anti-HCV in the ELISA among blood donors in Fukuoka prefecture. The prev-
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Table 6 Relative risks (with 95% confidence intervals) of HCC and LC for selected risk factors with and without adjustment for RIBA anti-HCV

<table>
<thead>
<tr>
<th>Factor</th>
<th>Anti-HCV not adjusted</th>
<th>Anti-HCV adjusted</th>
<th>Anti-HCV not adjusted</th>
<th>Anti-HCV adjusted</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indeterminate</td>
<td>11.8 (4.8-28.9)</td>
<td>-</td>
<td>10.4 (3.9-28.1)</td>
<td>-</td>
</tr>
<tr>
<td>Reactive</td>
<td>52.3 (23.9-114.3)</td>
<td>-</td>
<td>64.4 (27.4-151.4)</td>
<td>-</td>
</tr>
<tr>
<td>HBsAg positive</td>
<td>15.3 (6.1-38.1)</td>
<td>*</td>
<td>6.1 (2.2-17.2)</td>
<td>*</td>
</tr>
<tr>
<td>Positive history of blood transfusion</td>
<td>3.8 (2.0-7.0)</td>
<td>2.0 (0.9-4.7)</td>
<td>4.7 (2.5-8.8)</td>
<td>2.7 (1.2-6.2)</td>
</tr>
<tr>
<td>Positive history of heavy drinking</td>
<td>2.2 (1.3-3.8)</td>
<td>2.1 (1.0-4.4)</td>
<td>1.4 (0.7-2.8)</td>
<td>1.8 (0.7-4.3)</td>
</tr>
</tbody>
</table>

* Adjusted for sex, age, occupation, and years of education. For anti-HCV, individuals with ELISA-negative or RIBA-nonreactive results as the referent. For the other factors, those negative for the corresponding factor as the referent.

Table 7 Prevalence of serum anti-HCV in ELISA among blood donors in Fukuoka prefecture from November 1989 to March 1990

<table>
<thead>
<tr>
<th>Age groups (yr)</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of subjects</td>
<td>Anti-HCV(+)</td>
</tr>
<tr>
<td>16-19</td>
<td>7,668</td>
<td>27</td>
</tr>
<tr>
<td>20-29</td>
<td>9,434</td>
<td>96</td>
</tr>
<tr>
<td>30-39</td>
<td>9,888</td>
<td>147</td>
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<tr>
<td>40-49</td>
<td>7,221</td>
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</tr>
<tr>
<td>50-59</td>
<td>3,938</td>
<td>184</td>
</tr>
<tr>
<td>60-64</td>
<td>1,195</td>
<td>54</td>
</tr>
<tr>
<td>All ages</td>
<td>39,144</td>
<td>666</td>
</tr>
</tbody>
</table>

The prevalence of anti-HCV among elderly blood donors was considerably high, e.g., 4.7% for male donors ages 50 to 59 years, in comparison with the previous reports in other countries (32-35). HCV infection may be responsible for high HCC risk in this area.

We tentatively calculated the PAR (or etiological fraction, attributable fraction), i.e., the proportion of patients with a particular disease that can be attributed to a specified etiological factor, for HCV infection as well as chronic HBV infection. The derivation of PAR has been reported in detail by Greenland (36). When only RIBA-reactive results were regarded as significant in HCV infection, the PARs (and 95% CIs) of HCC and LC for HCV infection were estimated as 0.49 (0.39-0.60) and 0.50 (0.38-0.61), respectively, whereas the corresponding values for chronic HBV infection as indicated by positive HBsAg were 0.20 (0.11-0.28) and 0.09 (0.02-0.16), respectively. When RIBA-indeterminate results were considered as meaningful, the PARs of HCV infection were calculated as 0.62 (0.51-0.72) for HCC and 0.61 (0.49-0.72) for LC. This means that more than one-half of HCC or LC could be attributed to HCV infection. Thus, the possible role of HCV in the etiology of HCC and LC in Japan is extremely large and possibly more important than chronic HBV infection.

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