

## Characterization of the Humoral Immune Response against *Gnathostoma binucleatum* in Patients Clinically Diagnosed with Gnathostomiasis

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**Abstract.** Gnathostomiasis is an emerging systemic parasitic disease acquired by consuming raw or uncooked freshwater fish infected with the advanced third-stage larvae of *Gnathostoma* spp. This disease is endemic to the Pacific region of Mexico, and one of its etiologic agents has been identified as *Gnathostoma binucleatum*. We characterized the humoral immune response of patients clinically diagnosed with gnathostomiasis by detecting total IgM, IgE, and IgG class and subclasses against a crude extract of the parasite by Western blotting. Our results do not show differences in the antigens recognized by IgM and IgE. However, we found that the specific humoral immune response is caused mainly by IgG, specifically IgG4. We found that 43%, 65.2%, 54.1%, and 26.3% of the patients recognize the 37-kD, 33-kD, 31-kD, and 24-kDa antigens, suggesting that the 33-kD antigen is the immunodominant antigen of *G. binucleatum*.

### INTRODUCTION

Gnathostomiasis is a parasitic infection caused by advanced third-stage larvae (ADVL3) of the helminths *Gnathostoma* spp., which are seen mostly in tropical and subtropical regions. It is a food-borne zoonosis, and is endemic to areas where humans eat raw freshwater fish or shellfish, especially Thailand and other parts of Southeast Asia, Japan, and increasingly Latin America, particularly Mexico.<sup>1</sup> It is acquired by consuming raw or uncooked fresh-water fish infected with ADVL3,<sup>2</sup> in particular, swamp eels, catfish, sleeper perch, bream, Nile tilapia, butterfish, loaches, or snake-headed fish.<sup>1</sup> Epidemiologic studies have shown that more than 2,000 cases of this disease have been reported in Mexico since 1999, of which more than 500 were reported in the state of Nayarit.<sup>3</sup> Although 18 species of *Gnathostoma* have been recognized worldwide,<sup>4</sup> *G. binucleatum* is the only species found to infect humans in Mexico to date. However, other species may also infect humans.<sup>5</sup>

In humans, this disease is characterized by a combination of signs and symptoms caused by the parasite that include mechanical damage caused by migration of larvae, release of toxic substances, and an inflammatory reaction in the host.<sup>6</sup> Although neurologic and ocular symptoms have also been described, the most prevalent symptoms in Mexico are cutaneous, and no cases of invasion of the central nervous system have been reported.<sup>3</sup>

The definitive diagnosis of gnathostomiasis can be made by recovering the migrating larvae from skin lesions, but this procedure can be difficult because of the migratory behavior of this particular parasite.<sup>7</sup> However, it can be clinically diagnosed by obtaining a history of eating raw or partially cooked fish, intermittent subcutaneous or cutaneous migratory swelling, and eosinophilia.<sup>1</sup> Immunologic approaches have been developed to diagnose gnathostomiasis, including a cutaneous test, agglutination, immunofluorescence, enzyme-linked immunosorbent assay, and Western blotting.<sup>8–14</sup> Some of these tests use excretion–secretion products to detect antibodies in patients because specific functions attributed to the

excretion–secretion products of nematodes are invasion, and migration through host tissues, facilitation of feeding, and evasion of host immune responses.<sup>15</sup> However, for the development of these tests, previous characterization of the humoral immune response against the *Gnathostoma* spp. was necessary.

The IgG subclasses have been shown to provide improved specificity over the total IgG antibody array for the diagnosis of many parasitic infections, such as ascariasis,<sup>16</sup> echinococcosis,<sup>17</sup> leishmaniasis,<sup>18</sup> filariasis,<sup>19,20</sup> and gnathostomiasis caused by species of *Gnathostoma* other than *G. binucleatum*.<sup>21,22</sup> Therefore, the purpose of this study was to characterize the humoral immune response to a crude extract of *G. binucleatum* in patients with clinical diagnoses of gnathostomiasis to detect a possible antibody class or subclass that could be used in the diagnosis of gnathostomiasis.

### MATERIALS AND METHODS

**Patients and controls.** Serum samples from 73 patients with clinical diagnoses of gnathostomiasis who came to the Hospital General in Tepic, Nayarit, Mexico, were included in this study. Diagnoses were attained by using the following criteria: 1) subcutaneous or cutaneous migratory swelling, itching, and pain; and 2) a history of eating raw freshwater fish. In addition, serum samples from 20 healthy persons with no history of cutaneous or subcutaneous migratory swelling or previous symptoms compatible with migratory swelling, and no history of eating raw or uncooked fish; 14 samples from persons positive for intestinal parasites, and nine samples from persons negative for intestinal parasites at the time of the study were analyzed by using the formalin–ether concentration technique. Serum samples from two infants born at the same hospital and given a diagnosis of infection with *Toxoplasma gondii* (toxoplasmosis) were also included.

**Isolation of ADVL3 of *G. binucleatum*.** Advanced third-stage larvae of *G. binucleatum*, were isolated from fish (*Cathorops fuerthii*) obtained from local fishermen. To visualize areas of infection, thin fillets of fish musculature were compressed in polyethylene bags by using a metallic press and examined under a stereomicroscope. The ADVL3 were found free or encysted. Larvae were suspended in phosphate-buffered saline. Cysts were treated with artificial gastric fluid (0.259% HCl, 0.1% pepsin A) and isolated ADVL3 were then stored at –20°C.

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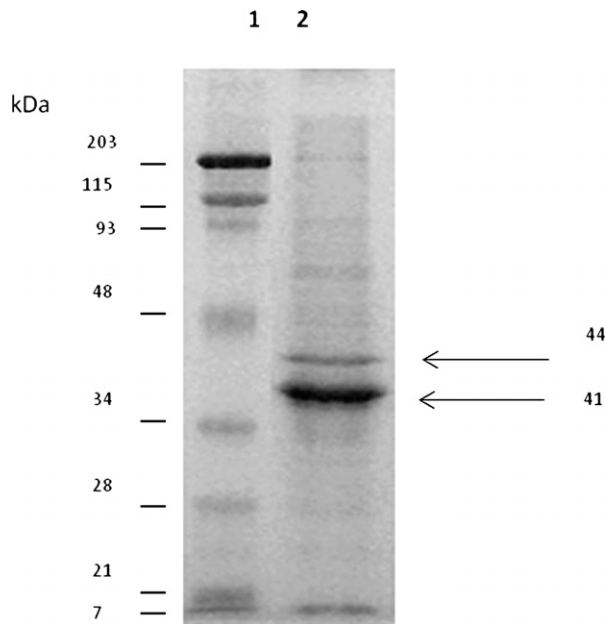


FIGURE 1. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Commaise blue staining of total proteins from isolated advanced third-stage larvae of *Gnathostoma binucleatum*. Lane 1, molecular mass markers; lane 2, crude extract. **Arrows** indicate the 44-kD and 41-kD antigens.

#### Sonicated crude extract of ADVL3 of *G. binucleatum*.

Approximately 500 ADVL3 were suspended in an extraction solution (1% Triton X-100, 0.05 mM tosyllysine chloromethylketone, 1 mM phenylmethylsulfonyl fluoride, 5  $\mu$ g/mL pepstatin A, and 41.54 mM EDTA), and a lysate was prepared by using a tissue homogenizer (PRO250; PRO Scientific, Inc., Oxford, CT) at 18,000 rpm for 5 minutes, followed by sonication at 20 kHz for 6 minutes in an ice bath (GE-130 Ultrasonic Processor; Cole-Parmer, Vernon Hills, IL). After centrifugation at 14,000 rpm (5810R; Eppendorf, Hamburg, Germany) for 15 minutes at 4°C, the supernatant was recovered, and protein quantification was carried out by using the bicinchoninic acid method (Pierce, Rockford, IL). The crude extract was then stored in aliquots at  $-20^{\circ}\text{C}$ .

**Molecular identification of *G. binucleatum*.** To confirm the identity of isolated parasites, genomic DNA from ADVL3 of *G. binucleatum* was obtained by using the genomic prep kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's instructions. Amplification of internal transcribed spacer 1 (ITS-1) and ITS-2 was conducted by using specific primers. For ITS-1 amplification, Lim1657 (forward) 5'-CTGCCTTTGTACACACCG-3', and ITS-RIXO (reverse) 5'-TGGCTGCGTTCTTCATCG-3' were used as reported.<sup>23</sup> For ITS-2 amplification, NEWS2 (forward) 5'-TGTGTCGATGAAGAACGCAG-3', and ITS2-RIXO (reverse) 5'-TTCTATGCTTTAAATTCAGGGG-3' were used.<sup>24</sup>

A polymerase chain reaction (PCR) was performed in a total volume of 50  $\mu$ L with 100 ng of genomic DNA, 4 mM  $\text{Mg}^{2+}$ , 10 mM dNTPs, 200 ng of each specific primer, and 2.5 units of *Taq* polymerase (Invitrogen, Carlsbad, CA). The amplification profile consisted of two minutes at min at  $94^{\circ}\text{C}$ ; 35 cycles of 30 seconds at  $94^{\circ}\text{C}$ , 30 seconds at  $54^{\circ}\text{C}$ , and 30 seconds at  $72^{\circ}\text{C}$ ; and a 7-minute elongation step at  $72^{\circ}\text{C}$ . The PCR products were purified by using the Concert Rapid PCR purification system (GIBCO, Gaithersburg, MD), according to the manufacturer's instructions, and then cloned into plasmid pCR2.1 by using the TA Cloning Kit (Invitrogen). These plasmids were used to transform competent *Escherichia coli* DH5 $\alpha$ E, and transformed bacteria were grown in selective Luria–Bertani medium containing ampicillin (25 mg/mL). One of the positive clones was selected, and expanded in Luria–Bertani medium containing ampicillin. Plasmids containing ITS-1 and ITS-2 fragments were purified using by using the Flexi-Prep Kit (Amersham Biosciences) and sequenced by using an ABI prism 310 genetic analyzer (Perkin-Elmer, Waltham, MA).

Sequences obtained were aligned with available sequences of species of *Gnathostoma* in the European Molecular Biology Laboratory Database and GenBank by using accession numbers for *G. binucleatum* (AY061741.1), *G. spinigerum* (Z97175.1), and *G. turgidum* (Z97176.1) and BLASTN 2.2.6 software ([www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE\\_TYPE=BlastNews](http://www.ncbi.nlm.nih.gov/blast/Blast.cgi?CMD=Web&PAGE_TYPE=BlastNews)). Sequences obtained were also compared with those reported from a *G. binucleatum* specimen obtained from a biopsy specimen.<sup>25</sup>

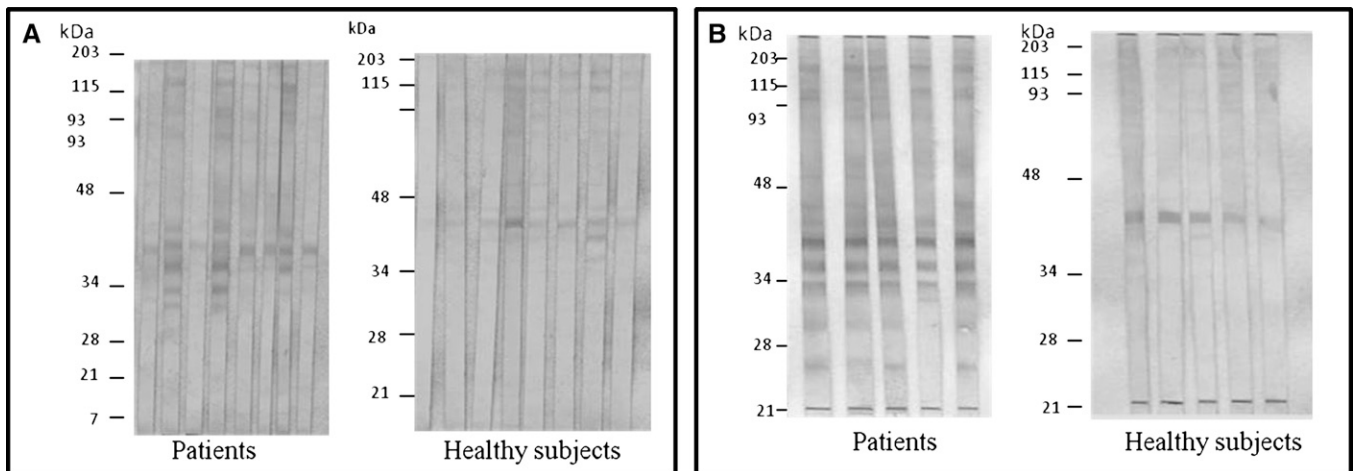


FIGURE 2. Representative Western blot strips showing protein patterns of a crude extract of *Gnathostoma binucleatum* recognized by antibodies from patients with gnathostomiasis and healthy subjects. **A**, Total immunoglobulins. **B**, IgG.

TABLE 1

Frequencies of *Gnathostoma binucleatum* antigens recognized by IgG subclasses from patients with a clinical diagnosis of gnathostomiasis\*

Relative molecular mass of antigen (kDa)	IgG1, no. (%)	IgG2, no. (%)	IgG3, no. (%)	IgG4, no. (%)
37	10 (13.8)	2 (2.7)	1 (1.3)	31 (43)†
33	4 (5.5)	2 (2.7)	0 (0)	47 (65.2)†
31	8 (11.1)	1 (1.3)	2 (2.7)	39 (54.1)†
24	0 (0)	0 (0)	1 (1.3)	19 (26.3)‡
21	2 (2.7)	0 (0)	0 (0)	0 (0)

\* Any of these bands were recognized by antibodies from the control group.

†  $P < 0.0001$ , by chi-square test.

‡  $P = 0.012$ , by chi-square test.

**Antibodies against ADVL3 of *G. binucleatum*.** Western blot analyses were conducted as described.<sup>26</sup> Briefly, 6.2 µg of total protein from the crude extract of ADVL3 of *G. binucleatum* were separated by electrophoresis in a 12% polyacrylamide gel, and blotted onto nitrocellulose sheets (0.45 µm; Amersham Biosciences) by using a miniprotean III transfer unit (Bio-Rad, Hercules, CA). After non-specific binding was blocked, nitrocellulose strips were incubated with each test serum (from patients or healthy persons) diluted 1:200 in blocking buffer. After washing, the nitrocellulose strips were incubated with a 1:100 dilution of peroxidase conjugate of either anti-human total immunoglobulins, anti-human IgG, anti-human IgE, anti-human IgM (all from Biosource, Camarillo, CA), or anti-human IgG subclasses (Zymed Laboratories, South San Francisco, CA) for 1 hour at 37°C. Antibody binding was developed by using 0.5 mg/mL of 3,3'-diaminobenzidine (Sigma, St. Louis, MO) and 0.03% H<sub>2</sub>O<sub>2</sub> in phosphate-buffered saline. Relative molecular masses of antigens specifically recognized by serum of patients were calculated in comparison to their retention value (Rf) by using standard molecular weight markers (Invitrogen).

**Data analysis.** Frequencies of recognition of different proteins in Western blot assays were analyzed by using the chi-square test and Minitab release 14.13 software ([www.minitab.com/en-US/products/minitab/default.aspx?WT.srch=1&WT.mc\\_id=SE003691](http://www.minitab.com/en-US/products/minitab/default.aspx?WT.srch=1&WT.mc_id=SE003691)). Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated as reported:<sup>27</sup> sensitivity = true-positive results/(true-positive results + false-negative results), specificity = true-negative results/(true-negative results + false-positive

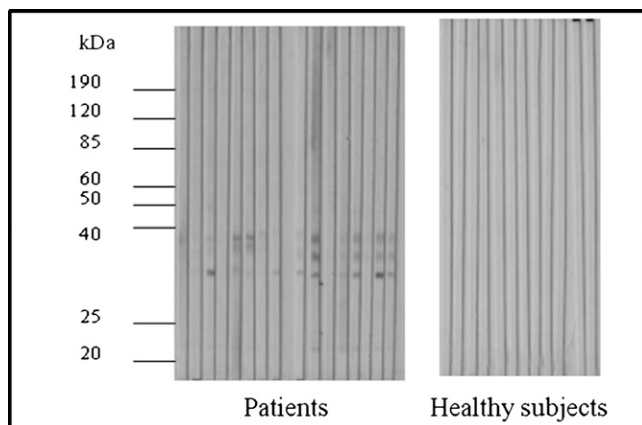


FIGURE 3. Representative Western blots strips showing protein patterns of a crude extract of *Gnathostoma binucleatum* recognized by IgG4 from patients with gnathostomiasis and healthy subjects.

TABLE 2

Sensitivity, specificity, PPV, and NPV for antigens recognized by IgG4 in patients with a clinical diagnosis of gnathostomiasis\*

Relative molecular mass of antigen (kDa)	Sensitivity, %	Specificity, %	PPV, %	NPV, %
37	43.06	100.00	100.00	32.79
33	65.28	100.00	100.00	44.44
31	54.17	100.00	100.00	37.74
24	26.39	100.00	100.00	27.40

\* PPV = positive predictive value; NPV = negative predictive value.

results), PPV = (true-positive results)/(true-positive results + false-positive results), and NPV = (true-negative results)/(true-negative results + false-negative results).

## RESULTS

**Molecular identification of *G. binucleatum*.** Our results showed that amplification fragments of ITS-1 and ITS-2 were 900 and 600 basepairs, respectively, which indicated that ADVL3 obtained were those of *G. binucleatum*.<sup>28</sup> Identity of parasites used in this study was confirmed by alignment of the 421-basepair fragment corresponding to ITS-2, which showed 100% identity with reported sequences of *G. binucleatum*<sup>25,28</sup> and 99.76% identity with sequence with sequence from the larva isolated from a biopsy specimen.<sup>25</sup>

The protein profile of the crude extract of ADVL3 of *G. binucleatum* showed bands ranging from 15 kDa to 190 kDa; prominent bands ranged from 35 kDa to 45 kDa (Figure 1). This crude extract was then used in Western blot analysis.

**Antigens of *G. binucleatum* recognized by serum of patients with gnathostomiasis.** When immunoglobulins were used to identify antigens of *G. binucleatum* recognized by serum of patients with gnathostomiasis, we found that bands with relative molecular masses greater than 40 kDa were recognized by serum from patients, and controls. One band with a molecular mass of either 37-, 33-, 31-, 24-, or 21-kDa was recognized by serum from patients, but not by healthy persons (Figure 2A) or patients given a diagnosis of toxoplasmosis.

When anti-human IgG was used, serum from patients with gnathostomiasis recognized bands of 37-, 33-, 31-, 24-, and 21-kDa, but serum from the control group did not recognize these antigens (Figure 2B). No differences in antigens recognized by patients and healthy persons were found with IgM, and IgE.

Twenty-four of 72, 5 of 72, 4 of 72, and 50 of 72 patients had IgG1, IgG2, IgG3, and IgG4, respectively, against *G. binucleatum* antigens (Table 1). When we analyzed IgG subclasses against 21–37-kDa bands, we found that few patients had IgG1, IgG2, or IgG3 against these bands. In contrast, 24-, 31-, 33-, and 37-kDa bands were strongly recognized by IgG4 from patients with gnathostomiasis (Figure 3 and Table 1). We found that serum samples from 65.2% of patients recognized the 33-kDa band, and serum samples from 94.0% of patients that had IgG4 against any antigen from *G. binucleatum* recognize the 33-kDa band. Thus, the 33-kDa antigen seems to be the immunodominant antigen in *G. binucleatum*. Serum samples from the control group did not show any reactivity with any antigen bands. For all subclasses, no differences in recognition of bands were found between healthy persons who were positive or negative for intestinal parasites.

Results for 24-, 31-, 33-, and 37-kDa antigens recognized by IgG4 from patients with gnathostomiasis are shown in Table 2. In all cases, the specificity and PPV were 100% because serum



samples from healthy persons did not recognize any of these bands. The highest sensitivity and specificity were obtained for the 33-kD antigen (65.28% and 44.4%, respectively).

## DISCUSSION

Human gnathostomiasis is a disease caused by *Gnathostoma* spp. especially in tropical countries and is a serious public health problem.<sup>1</sup> Although definitive diagnosis of the disease is made by recovering the migrating larvae from skin lesions, this procedure can be difficult because of the migratory behavior of the parasite.<sup>7</sup> Characterization of the immune response against *Gnathostoma* spp. has been useful in the development of a diagnostic tool, as reported for *G. spinigerum*.<sup>21,22,29</sup> However, in the Pacific region of Mexico, species of *Gnathostoma* other than *G. spinigerum* and *G. doloresi* are responsible for the disease, specifically *G. binucleatum*.<sup>25</sup> Thus, antigens and immune responses observed in patients infected with other species of *Gnathostoma* could be different for those observed with patients infected with *G. binucleatum*.

To characterize the immune response against *G. binucleatum*, the first antigenic screening was conducted by using serum samples from patients with gnathostomiasis and total human immunoglobulins and analyzed by using Western blotting. As shown in Figure 2A, both groups included in this study had antibodies against antigens greater than 40 kD. These results are consistent with those reported for other species of *Gnathostoma* because none of these bands have been reported as being specifically recognized by serum samples from patients infected with other species of *Gnathostoma*.<sup>11,30,31</sup> However, the 21–37-kD antigens might be involved in the immune response against *G. binucleatum* because these antigens were recognized by most patients but not by healthy persons. Moreover, when IgG was used, the same profile of recognition was observed (Figure 2B). It has been reported that patients infected with *G. spinigerum* have IgG that recognized antigens of 20.1 kD to > 94 kD.<sup>21</sup> However, it has also been reported that total IgG against *G. spinigerum* shows cross-reactivity with other parasites.<sup>22</sup>

We investigated whether antigens of *G. binucleatum* recognized by total IgG showed any differences in recognition when each IgG subclass was tested by Western blotting. As shown in Table 1 patients with gnathostomiasis caused by *G. binucleatum* showed IgG1, IgG2, or IgG3 responses against antigens of this parasite. Contrasting reports have been published about IgG subclasses and antigens of *G. spinigerum* recognized by patient serum. Laummaunwai and others<sup>21</sup> reported that a 24-kD antigen from *G. spinigerum* was recognized by IgG1 from 66.7% of infected patients and proposed that the 24-kD antigen could be used in the diagnosis of gnathostomiasis. In contrast, another study reported that IgG4, but not IgG1, IgG2, or IgG3, from patients infected with *G. spinigerum* recognize a 21-kD antigen and that 30-, 32-, 33-, and 34-kD antigens were weakly recognized.<sup>32</sup> We report that 21–37-kD antigens are recognized by IgG4 from patients with gnathostomiasis (Figure 3), and that the 33-kD antigen was recognized by 47 (65.1%) of 72 patients but weakly recognized by IgG1 and IgG2 ( $P = 0.001$ ). All of these antigens were recognized by serum from the control group.

These results indicate that the major component of the IgG response to *G. binucleatum* is IgG4, which is in contrast to data reported for *G. spinigerum*, which showed that IgG1 and IgG2 were the major components of the immune response.<sup>22</sup> More-

over, the immunodominant antigen from *G. binucleatum* is likely the 33-kD antigen.

In this context, the 24-, 31-, 33-, and 37-kD antigens are recognized by IgG4 from patients with gnathostomiasis but not by healthy controls. As shown in Table 2, the highest values for sensitivity, specificity, PPN, and NPV were for the 33-kD antigen. In all cases, specificity and the PPV were 100% because the healthy controls did not recognize any of these bands. However, it is necessary to validate the usefulness of these antigens, alone or in combination, as a diagnostic tool.

Published data indicated that IgE levels in serum of patients with gnathostomiasis caused by *G. spinigerum* was 10-fold higher compared with that in healthy controls.<sup>33,34</sup> We found that patients and controls recognize only the unspecific 44-kDa antigen, and that no differences in IgM levels were observed for antigens recognized by serum from patients and healthy controls.

Our results suggest that the immune response against *G. binucleatum* is different from that against other *Gnathostoma* species, such as *G. doloresi*, and *G. spinigerum*,<sup>13</sup> which have been found to infect humans in Mexico. In addition, characterization of antigens and antibodies involved in the immune response in patients could help establish a specific diagnostic test for disease caused by *G. binucleatum*, as has been developed for *G. spinigerum*.<sup>22</sup> Moreover, the role of differences in the humoral immune response observed and the potential of the 33-kD antigen and IgG4 for development of a diagnostic tool should also be investigated.

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