BASIC RESEARCH

Matrix metalloproteinase-9 expression is associated with the presence of *Chlamydia pneumoniae* in human coronary atherosclerotic plaques

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Objective: To investigate the association between *Chlamydia pneumoniae* and matrix metalloproteinase-9 (MMP-9) in atherosclerotic plaques.

Design: 31 coronary atherosclerotic plaque specimens were studied by immunohistochemistry, polymerase chain reaction (PCR), and reverse transcription PCR for the presence of *C pneumoniae* antigen and genomic DNA, and of MMP-9 protein and transcripts.

Results: Immunohistochemical analysis identified a strong association between the presence of *C pneumoniae* antigen and production of MMP-9 in coronary atherosclerotic plaques (p = 0.001). Furthermore, analysis of the intralesional amount of *C pneumoniae* and MMP-9 indicated an increased number of cells positive for MMP-9 in arterial sections that had increased *C pneumoniae* positivity (p < 0.05).

Conclusions: This study provides evidence of an association between expression of MMP-9 and the intravascular presence of *C pneumoniae* and may suggest a potential pathological mechanism whereby *C pneumoniae* may contribute to the progression of coronary atherosclerosis.

A therosclerosis is now generally considered to be an inflammatory disease, but the underlying mechanisms of induction and progression of the lesions are still unclear.[1] The role of infectious agents in atherogenesis has been studied extensively with *Chlamydia pneumoniae* evoking particular interest in recent years.[2]

A relation between *C pneumoniae* and atherosclerosis has been postulated based mainly on the presence of the organism in plaques, experimental studies in animals,[3–5] and results of pilot antibiotic trials in patients.[6–11] Opinion, however, is divided as to the role of *C pneumoniae* in the pathogenesis of atherosclerosis, as recent studies have yielded controversial results.[12 13]

Despite the dichotomy of opinion concerning *C pneumoniae* and atherosclerosis, it is generally accepted that chronic infection of the vascular wall may initiate or exacerbate an ongoing inflammatory response.[1] A key component of inflammation is the macrophage population. Macrophages infiltrate early atherosclerotic lesions accumulating oxidised low density lipoprotein. *C pneumoniae* has been identified within macrophages and smooth muscle cells of atherosclerotic tissue[14] and shown to be able to infect and survive in human macrophages, arterial smooth muscle cells, and endothelial cells.[14 15] In addition, *C pneumoniae* induces foam cell formation by macrophages.[15 16]

Matrix metalloproteinases (MMPs) are proteolytic enzymes that are involved in the degradation of matrix components. The 92 kDa gelatinase MMP-9 is often expressed in atherosclerotic plaques by macrophages and smooth muscle cells[17] and degrades the matrix components in the fibrous cap.[18] Plaque shoulders, regions of foam cell accumulation, and microvasculature have increased expression of MMP-9.[19] It is suggested that MMP-9 has a role in unstable angina[17] and vascular remodelling.[18] Increased expression of MMP-9 has been associated with the formation of intimal hyperplasia and carotid artery remodelling in a mouse blood flow cessation model.[20] *C pneumoniae* and its heat shock protein-60 have been shown to induce expression of MMP-9 and tumour necrosis factor α in mouse macrophages in vitro[21] and more recently in human monocyte derived macrophages in vitro.[22]

The objective of the present study was to investigate the association between intralesional *C pneumoniae* infection and MMP-9 production. In addition the extent of macrophage infiltration and foam cell accumulation were examined and correlated with cellular localisation of both *C pneumoniae* and MMP-9.

METHODS

Patients and samples

Human coronary endarterectomy specimens were obtained from 24 patients (21 men, mean age 63 (8) years) requiring elective urgent coronary artery bypass surgery. A further seven human coronary artery samples were obtained from the explanted hearts of patients undergoing cardiac transplantation (seven men, mean (SD) age 44 (11) years). Venous samples from restenotic bypass grafts were excluded. Samples were stored at 4°C until being processed on the same day as surgery. The study protocol was approved by the local ethics committee and all participants gave written informed consent.

Histology and immunohistochemistry

Serial 5 μm cryostat sections of atherosclerotic coronary arteries were analysed by immunofluorescence double labelling. Sections were cut, air dried on multispot slides, and frozen until use. For immunohistochemical analysis, sequential sections were double stained for *C pneumoniae* and CD68, MMP-9 and CD68, negative control and α smooth muscle cell actin, or CD31 and negative control. Sections were initially blocked with rabbit serum (Dako) containing four drops/ml avidin solution (avidin/biotin blocking kit, Vector Labs) for

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Abbreviations: MMP, matrix metalloproteinase; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction

15 minutes, washed briefly with phosphate buffered saline, and incubated with the primary antibodies. The primary antibodies, prepared in phosphate buffered saline containing four drops/ml biotin (Avidin/Biotin blocking kit, Vector Labs), were mouse anti-*C pneumoniae* major outer membrane protein (clone RR402, Dako), mouse anti-MMP-9 (clone 56–2A4, Oncogene Biosciences), or mouse IgG3 (Sigma) used as a negative control. After an overnight incubation at 4°C, the sections were washed and incubated with biotin conjugated rabbit anti-mouse immunoglobulins (Dako) followed by streptavidin/fluorescein isothiocyanate (Dako).

Sections were then labelled with antibodies to the macrophage marker CD68 (Dako), α smooth muscle cell actin (Sigma), or endothelial cell CD31 (Dako) for one hour at room temperature, followed by incubation with biotin conjugated rabbit anti-mouse immunoglobulins (Dako) and streptavidin/7-amino-4-methylcoumarin-3-acetate (Jackson Labs). Immunostaining with the anti-*C pneumoniae*, anti-MMP-9, and anti-CD68 and the presence of foam cells were assessed by using the following grades: 1+, 2+, 3+, and 4+, corresponding to immunoreactivity in 1–10, 11–20, 20–30, and over 30 cells per × 400 microscope field, and 0 for negative sections. To determine the stage of atherosclerosis, sections were analysed by haematoxylin and eosin staining and the plaques were classified according to the recommendation published by Stary and colleagues.[23]

To validate the findings and rule out the possibility of false positive specimens caused by artefactual staining, 18 of 22 *C pneumoniae* positive specimens were re-examined for chlamydial antigen with the mouse *C pneumoniae* monoclonal antibody (RR402) and a further mouse *C pneumoniae* monoclonal antibody (clone A57-B9, Affinity Bioreagents Inc) specific for the 60 kD heat shock protein of *C pneumoniae*.

Positive control slides of *C pneumoniae* infected Hep-2 cells (Dako) were also analysed.

Detection of *C pneumoniae* DNA by polymerase chain reaction

C pneumoniae genomic DNA was detected by nested polymerase chain reaction (PCR). Briefly, 5 mm long artery sections were digested for three hours at 55°C in 5% sodium dodecyl sulfate and 400 μg/ml proteinase K in tris-EDTA buffer pH 8.0 followed by phenol chloroform DNA extraction and ethanol precipitation. After DNA quantification, 500 μg of DNA was subjected to nested PCR with oligonucleotide primers specific for the major outer membrane protein of *C pneumoniae* (first round: sense, 5′-TTACAAGCCTTG CTTGTAGG-3′; antisense, 5′-GCGATCCCAAATGTTTAAGGC. Second round: sense, 5′-TTATTAATTGATGGTACAATA-3′; antisense, 5′-ATCTACGGCAGTAGTATAGTT-3′) according to procedures described elsewhere.[24] The resulting 207 base pair amplicons were analysed by agarose gel electrophoresis. Amplification of the housekeeping gene myoglobin served as a control confirming the integrity of the extracted DNA.[25]

Detection of MMP-9 transcript by reverse transcription PCR

MMP-9 transcription was analysed by reverse transcription (RT) -PCR as described.[22] Briefly, total RNA was isolated from 5 mm long artery sections with ultrapure TRIzol (Gibco) and reverse transcribed into cDNA with the Superscript II preamplification kit (Gibco). The cDNA was subjected to PCR with oligonucleotide primers specific for human MMP-9 (sense, 5′-CACTGTCCACCCCTCAGAGC-3′; antisense, 5′-GCCACTTGTCGGCGATAAGG-3′).[26] To confirm the integrity of the extracted RNA, transcription of the housekeeping gene

Table 1 Summary of immunohistochemical analysis, PCR, RT-PCR, and plaque grade data

Patient	Age (years)/sex	Macrophage grade	Foam cell grade	*C pneumoniae* PCR	MMP-9 RT-PCR	Stage of atherosclerosis*	Immunostaining *C pneumoniae*	Immunostaining MMP-9
1	58/M	4+	1+	–	+	VIa	3+	2+
2	67/M	1+	0	+	+	VIc	1+	1+
3	63/M	2+	2+	–	+	IV	4+	2+
4	59/M	2+	0	–	+	Vc	1+	1+
5	51/M	3+	1+	–	+	Vc	2+	2+
6	53/M	3+	2+	–	+	Vb	0	3+
7	70/F	4+	3+	–	+	Va	3+	3+
8	37/M	1+	0	–	+	II	4+	4+
9	66/M	2+	1+	–	+	Vb	3+	2+
10	49/M	2+	3+	–	+	Vb	0	2+
11	60/M	2+	3+	–	+	Vb	1+	1+
12	69/M	2+	3+	–	–	Vb	0	0
13	52/M	4+	3+	–	+	Vc	0	1+
14	58/M	3+	NT	–	+	IV	2+	3+
15	35/M	1+	NT	–	+	I	0	2+
16	27/M	2+	2+	+	+	Va	2+	2+
17	48/M	1+	0	–	–	Vb	0	0
18	56/F	4+	4+	–	NT	VIa	0	2+
19	55/M	3+	1+	–	+	Va	3+	1+
20	53/M	2+	2+	–	NT	IV	3+	2+
21	75/M	1+	1+	–	NT	Vc	2+	2+
22	68/M	3+	2+	–	+	Va	2+	3+
23	71/M	2+	0	+	+	Va	1+	2+
24	65/M	2+	1+	–	–	III	3+	1+
25	70/M	2+	0	–	+	Vb	2+	1+
26	72/M	3+	2+	–	+	Vc	2+	2+
27	50/M	3+	3+	–	+	Vb	4+	2+
28	59/F	1+	0	–	–	Va	0	0
29	69/M	2+	1+	+	+	Va	2+	1+
30	76/M	3+	1+	–	+	Vc	2+	1+
31	64/M	2+	3+	–	–	Vc	0	0

Immunohistochemistry data shown as grades 0 to 4+.
*Classified according to the recommendations by Stary *et al.*[25]
C pneumoniae, Chlamydia pneumoniae; F, female; M, male; MMP-9, matrix metalloproteinase-9; NT, not tested; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction.

glyceraldehyde-3-phosphate dehydrogenase was analysed as a control (sense, 5'-ACCACAGTCCATGCCATCAC-3'; antisense, 5'-TCCACCACCCTGTTGCTGTA-3').[22] The resulting PCR amplicons were analysed by agarose gel electrophoresis.

Statistical analysis

Statistical analysis was carried out with the SPSS version 10 statistics package (SPSS Inc, Chicago, Illinois, USA). Results were analysed as categorical data with the χ^2 test and Fisher's exact test where necessary. A probability value of $p < 0.05$ was considered significant.

RESULTS

Immunohistochemical analysis of human coronary artery specimens

Analysis of human coronary atherosclerotic lesions by immunohistochemical double staining showed an association between intralesional presence of *C pneumoniae* and increased expression of MMP-9. Immunoreactive chlamydial antigen was detected in 22 of 31 specimens (71%) and immunoreactive MMP-9 was detected in 27 of 31 specimens (86%) (table 1). The positive chlamydial staining was re-examined and confirmed in 18 available specimens with the *C pneumoniae* antibody and a second monoclonal antibody to a distinct chlamydial antigen (fig 1). Incubation of specimens with negative control mouse antibodies resulted in no staining. The four samples that were negative for MMP-9 were also negative for *C pneumoniae* (table 1). These data support that detection of MMP-9 in coronary atherosclerotic plaques is associated with the intralesional presence of *C pneumoniae* ($p = 0.001$). Furthermore, analysis of the intralesional amount of *C pneumoniae* positivity and MMP-9 production by the grading system outlined above indicated that the number of cells positive for MMP-9 was apparently increased in arterial sections that had a higher level of *C pneumoniae* positivity. Analysis of these data showed that there was a significant association between the extent of chlamydial infection and the amount of MMP-9 production ($p < 0.05$).

To determine the cellular localisation of *C pneumoniae* and MMP-9, double immunostaining was carried out with *C pneumoniae* antibody or anti-MMP-9 antibody with CD68 antibody, and sequential sections were immunostained with α smooth muscle cell actin and anti-CD31 antibodies. Chlamydial antigen co-localised with macrophages (fig 1), smooth muscle cells, and endothelial cells. MMP-9 was observed in macrophages, smooth muscle cells, and endothelial cells (figs 2 and 3). Both MMP-9 and *C pneumoniae* were often observed within macrophage rich regions in the plaque (fig 2) and in areas of extensive smooth muscle cell infiltration such as the shoulder regions of the plaque. Analysis of serial sections showed that MMP-9 co-localised with *C pneumoniae* in 13 of 19 specimens (68%).

Immunohistochemical analysis of macrophage infiltration and foam cell accumulation by CD68 staining (table 1) showed that there was a significant correlation between the extent of macrophage infiltration and foam cell accumulation ($p < 0.05$). There was, however, no significant association between macrophage infiltration and presence of intralesional *C pneumoniae* or MMP-9. There was also no significant association between foam cell accumulation and *C pneumoniae* infection.

Identification of *C pneumoniae* genomic DNA and MMP-9 transcripts in human coronary artery specimens

Nested PCR analysis identified the presence of *C pneumoniae* genomic DNA in four of 31 specimens (13%). Although this detection rate is lower than that of the immunohistochemical analysis, all four samples that were positive by nested PCR were also positive by *C pneumoniae* immunostaining. RT-PCR analysis of total cellular RNA showed the presence of MMP-9 transcripts in 16 of 28 specimens (57%). Comparison of the RT-PCR and immunohistochemical detection of MMP-9 showed a significant linear trend ($p = 0.024$). All four specimens positive for *C pneumoniae* by nested PCR showed MMP-9 production by both RT-PCR and immunohistochemistry.
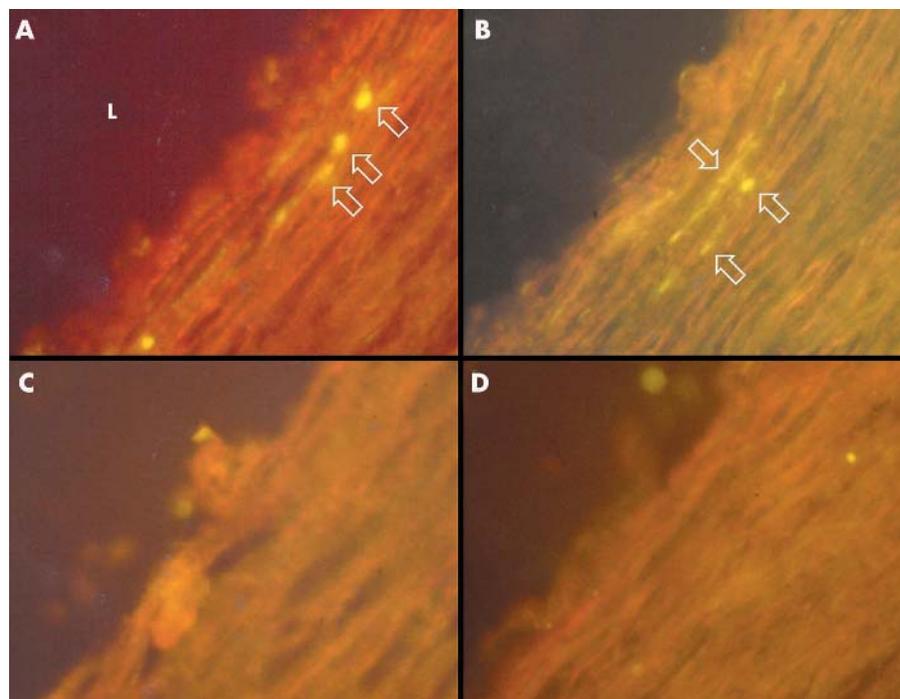

Figure 1 Immunohistochemical staining of 5 μm sections of a coronary endarterectomy specimen with monoclonal antibodies to two distinct antigens of *Chlamydia pneumoniae*. Sequential sections stained with (A) fluorescein isothiocyanate (FITC) labelled *C pneumoniae* major outer membrane protein antibody (clone RR402) and (C) an IgG3 isotype control antibody. Sequential sections of the same specimen stained with (B) FITC labelled *C pneumoniae* heat shock protein-60 antibody (clone A57-B9) and (D) an IgG1 isotype control antibody. Arrows indicate positively stained cells. L, artery lumen. (Original magnification ×400).
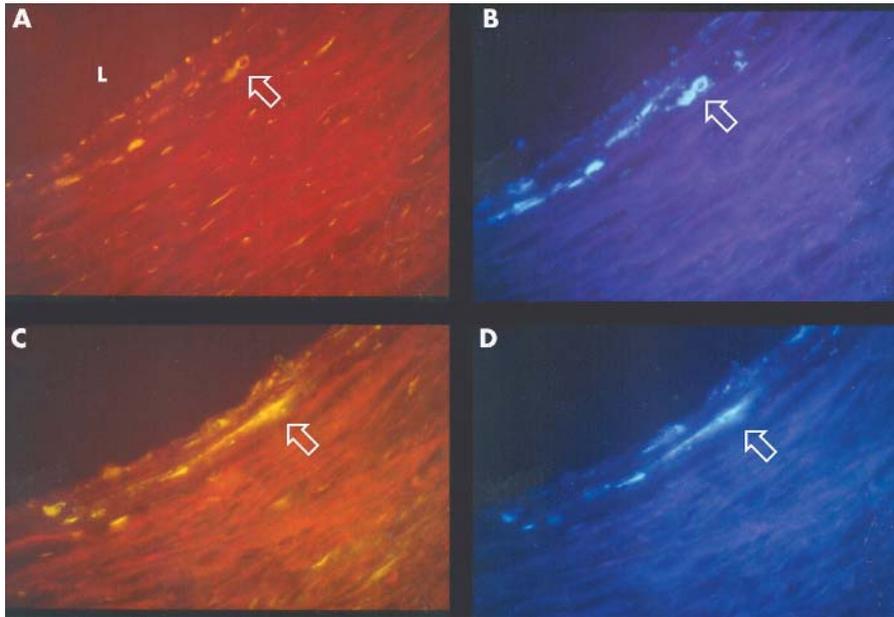
Figure 2 Double immunohisto-chemical staining of 5 μm sections of a coronary endarterectomy specimen showing co-localisation of matrix metalloproteinase-9 (MMP-9) and *C pneumoniae* in the arterial wall. Sequential sections stained with FITC labelled antibodies to (A) *C pneumoniae* (RR402) and (C) MMP-9. (B, D) The same sections stained with 7-amino-4-methylcoumarin-3-acetate (AMCA) labelled CD68 antibody. Arrows indicate positively stained cells. (Original magnification ×400).
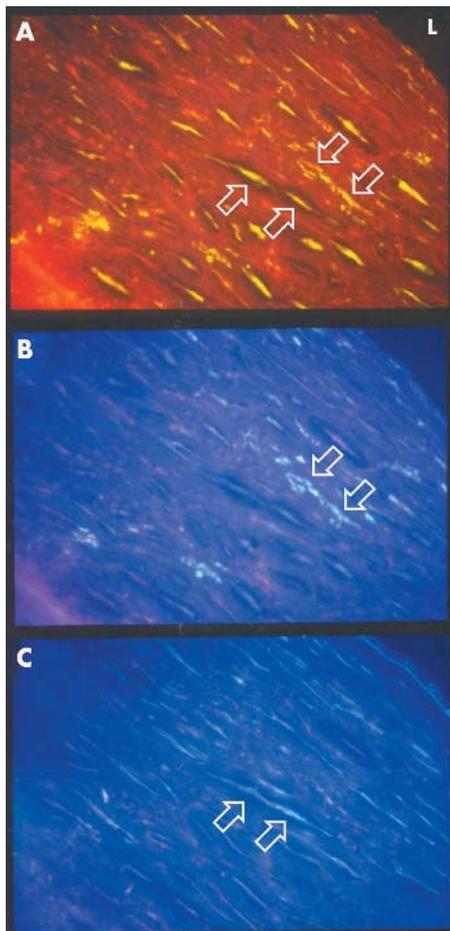

Figure 3 Double immunohistochemical staining of 5 μm sections of a coronary endarterectomy specimen showing smooth muscle cells and macrophages expressing MMP-9. (A) FITC staining of MMP-9. (B) AMCA staining of CD68. (C) AMCA staining of α smooth muscle cell actin. Upward arrows indicate cells positive for α smooth muscle cell actin and MMP-9 stain. Downward arrows indicate cells positive for CD68 and MMP-9 stain. (Original magnification ×400).

DISCUSSION

This study provides evidence of an association between increased MMP-9 production and the presence of *C pneumoniae* in human coronary atherosclerosis and may provide evidence for a potential mechanism by which the bacteria may accelerate or exacerbate coronary atherosclerotic disease.

C pneumoniae has been associated with atherosclerosis and coronary artery disease, although the mechanism by which the bacterium may affect disease initiation or progression is poorly understood. Recent evidence shows that *C pneumoniae* can infect, survive in, and stimulate macrophages, smooth muscle cells, and endothelial cells,[27 28] resulting in foam cell formation and MMP-9 production by macrophages in vitro.[15 21 22 29 30] MMP-9 is an important contributing factor to atherosclerotic disease and is upregulated in atherosclerotic plaques, particularly during remodelling, in unstable plaques and in macrophage, foam cell, and smooth muscle cell rich areas. It is suggested that MMP-9 is associated with exacerbation of atherosclerosis and advancement of disease by breaking down matrix components and by inducing instability and rupture of the fibrous cap.

The data described here suggest that there is a strong association between the presence of *C pneumoniae* and MMP-9 production. A significant association was found between the extent of intralesional *C pneumoniae* and the amount of MMP-9 production. When serial sections of tissue were examined, *C pneumoniae* and MMP-9 were co-localised to the same regions of the plaque. There was no evidence in these specimens to suggest that *C pneumoniae* is associated with increased macrophage infiltration or foam cell formation. As MMP-9 is expressed by macrophages as well as smooth muscle cells it was not possible to ascribe the increase in MMP-9 expression to macrophage infiltration alone. The use of immunohistochemical as well as molecular techniques allowed confirmation of the association between infection with *C pneumoniae* and upregulation of MMP-9 transcription. Although a proportionally lower number of samples was found positive by *C pneumoniae* PCR, this is a finding well documented by others[2] and may reflect rapid degradation of chlamydial genomic DNA and persistence of chlamydial membrane proteins within the plaque, a phenomenon that has been shown to occur with the use of in situ DNA nick end

labelling (terminal deoxynucleotidyl transferase-mediated dUDP nick end labelling).[31]

The results of the work described here are in agreement with previously published findings. *C pneumoniae* has been shown to increase the capacity of macrophages to produce MMP-9 by stimulating the expression of this enzyme in human monocyte derived macrophages.[22] In mouse macrophages, *C pneumoniae* and its 60 kDa heat shock protein stimulate tumour necrosis factor α and MMP-9 production.[21] *C pneumoniae* has been shown to activate the nuclear factor κB pathway in vascular smooth muscle cells and endothelial cells.[32] In a study by Song and colleagues,[33] in four atherosclerotic aortic tissue specimens immunoreactivity for MMP-9 and cyclooxygenase-2 co-localised with immunoreactivity for *C pneumoniae*. Choi and colleagues[34] recently observed expression of MMP-9 co-localised with *C pneumoniae* staining in diseased aorta and carotid artery specimens.

In contrast however, Petersen and colleagues[35] did not observe any association between a positive DNA test for *C pneumoniae* and activity of MMP-2 and MMP-9 in aortic aneurysms.[35] However, the authors used separate blocks of tissue for detection of *C pneumoniae* DNA and MMP activity. The advantage of the present study is that the MMP-9 and *C pneumoniae* could be detected in sequential 5 μm sections by immunohistochemistry.

In conclusion, the data presented here provide evidence of an association between increased MMP-9 production and intralesional presence of *C pneumoniae* in coronary atherosclerosis and may provide increased evidence for a potential mechanism by which the bacterium may accelerate or exacerbate atherosclerotic disease.

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