MAJOR ARTICLES

Detection of Circulating *Chlamydophila pneumoniae* in Patients with Coronary Artery Disease and Healthy Control Subjects

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Background. There is a long history of research suggesting that *Chlamydophila pneumoniae* is associated with coronary artery disease (CAD). *C. pneumoniae* in peripheral blood mononuclear cells (PBMCs) could serve as a risk factor for CAD if respiratory infection with *C. pneumoniae* spreads to atherosclerotic plaques through PBMCs or if infected plaques shed *C. pneumoniae*–laden PBMCs into the circulation.

Methods. PBMCs were collected from 86 case patients with abnormal coronary angiogram findings and from 91 age- and gender-matched healthy control subjects. The healthy control subjects were strictly defined as not having atherosclerosis on the basis of absence of both clinical atherosclerotic disease and traditional risk factors for CAD. PBMCs were probed for the presence of *C. pneumoniae* nucleic acid by 2 separate real-time polymerase chain reaction (PCR) assays that used primers for outer membrane protein A *(ompA)* and 16S ribosomal RNA. *C. pneumoniae* serologic findings were determined for both case patients and control subjects.

Results. Despite serologic findings indicating past exposure to *C. pneumoniae* (immunoglobulin G titer, $\geqslant$1:16) in 74% of case patients with CAD and control subjects, no *C. pneumoniae* DNA or RNA was detected in PBMCs from any of the case patients or control subjects, including a subset of 42 participants (18 with CAD) who had samples obtained serially over 8 months. Multiple laboratory controls, including controls for inhibition of PCR, produced expected results.

Conclusions. The uniformly negative results with use of highly sensitive methods are in contrast to much of the published literature. Probing of PBMCs for the genes of *C. pneumoniae* does not appear useful as a noninvasive way of detecting the presence of *C. pneumoniae* in atheromatous lesions.

The possibility that *Chlamydophila pneumoniae* might initiate, accelerate, or cause the complications of atherosclerosis has stimulated a large investigative effort [1]. Epidemiologic studies conducted as early as the 1980s suggested a correlation between the presence of antibodies to *C. pneumoniae* and coronary artery disease (CAD) [2]. Subsequently, a role for *C. pneumoniae* was suggested by lipid biochemical studies, acceleration of atherosclerosis in animal models, and reports of *C. pneumoniae* in human vascular atheroma [2–4]. Of interest, several human secondary prevention antibiotic trials failed to demonstrate a treatment benefit [4].

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These negative results led to a period of skepticism. Reviews of previous studies revealed significant heterogeneity in methodology. For example, at the time of these clinical trials, there were 18 published in-house PCR assays for the detection of *C. pneumoniae*. In response to the problem of heterogeneity, in 2001, the Centers for Disease Control and Prevention and the Canadian Laboratory Centre for Disease Control recommended uniformity of laboratory methods [5]. A recent review indicates reports of multiple additional assays, many in which PCR methods are poorly described, making extrapolation between studies impossible, and to date, there is no standardized PCR assay approved by the US Food and Drug Administration [6].

Despite appropriate criticisms of the methods used for serologic examination, PCR, and other detection techniques, interest continues because of several observations [6–8]. In experimental models of *C. pneu-*

moniae pneumonia, data support transportation of *C. pneumoniae* from the lung to vascular cells through PBMCs [9–12]. *C. pneumoniae* in PBMCs spontaneously enter into an altered vegetative but viable antibiotic-resistant "persistent" state [13, 14]. This may have contributed to the negative results of the secondary prevention antibiotic trials [4]. In addition, recent data have revealed *C. pneumoniae* polymorphisms that display increased tropism for vascular endothelial cells [15]. There continue to be reports of detection of *C. pneumoniae* in PBMCs from patients with atherosclerotic vascular disease [4]. However, the reported range of positivity is very wide, from 0% to 100%, likely because of significant variability in laboratory methods [7] and in definitions of control populations. In addition, several studies have noted potential for seasonal variation in rates of detection of *C. pneumoniae* DNA in PBMCs, with most positive findings occurring for samples obtained during the winter months [7, 16–21]; few studies have been designed to address this question [20, 21]. Other studies have suggested that the persistence of *C. pneumoniae* DNA in PBMCs may vary over time [21], and serial determinations may be necessary to assess infection.

Therefore, we designed this study to investigate the relationship between the presence of *C. pneumoniae* in PBMCs and CAD. Our study differs from prior studies in several important ways, as follows: (1) use of our precise definition of 2 distinct groups of patients with and without heart disease, (2) use of PCR methods that meet published criteria for sensitivity and specificity, and (3) use of serial samples from individual patients that were obtained over 3 seasons to assess potential seasonal effect or variability over time.

PATIENTS, MATERIALS, AND METHODS

Clinical procedures. We estimated that a sample size of 80 participants in each group would demonstrate a statistically significant increase in the prevalence of *C. pneumoniae* nucleic acid in PBMCs with 80% power if the prevalence was ~2.5% in the control group and ~15% in the case group. This study was approved by the Providence Health System Institutional Review Board, and all participants provided informed consent.

All patients aged 40–85 years who received their medical care in the Providence Health System in Portland, Oregon, were eligible for this case-control study. Case patients with CAD were age- and gender-matched with healthy control subjects.

A case patient was defined as a patient with CAD. CAD was defined by abnormal findings of an angiogram performed from 1 January 2005 through 1 October 2006 that documented >50% blockage of ⩾1 major coronary vessels. Case patients were identified using an internal database of angiograms performed at Providence Portland Medical Center or at Providence St. Vincent's Medical Center. Case patients were randomly selected and recruited by phone. Eight-six case patients participated.

A control subject was defined as a patient with no clinical evidence of CAD and no risk factors for CAD. Our definition of no risk factors required no history of atherosclerotic disease and an absence of current tobacco use, hypercholesterolemia or cholesterol-lowering medication use, hypertension or antihypertensive medication use, diabetes, and renal disease. Control subjects were identified using an internal database of a large primary care group practice (Providence Medical Group) that contains >300,000 patients. The database was searched by diagnostic code. Potential control subjects were recruited from phone lists generated using a random number generator. Control subjects were age- and gender-matched with case patients by 5-year age increments. Control subjects were further screened for inclusion criteria by phone and at their first study visit. Exclusion criteria for all patients included failure to meet the inclusion criteria above or inability to sign informed consent.

Initial collection of samples from patients occurred during the fall of 2006. Patients completed full informed consent forms, and study personnel collected medical data and obtained blood samples. All case patients and control subjects were invited to return for obtainment of serial blood samples every 2 months during the study period (5 times during October 2006–June 2007 for case patients and 4 times during November 2006–June 2007 for control subjects). Of the 177 patients, 42 (18 case patients and 24 control subjects) had all serial samples obtained.

Laboratory methods. We collected 5–10 mL of whole blood in sodium heparin tubes at each study visit. Samples were blinded. PBMCs were immediately isolated from plasma with use of hypaque-ficoll (6% Ficoll in PBS), were washed in PBS, and were collected after centrifugation at 383 *g* for 15 min. PBMCs were resuspended in 400 μL of 0.9% saline and were stored in 2 aliquots at −70°C for later extraction. One 20-μL aliquot of PBMCs was placed in bioMérieux robot for nucleic acid extraction and was then suspended in 25 μL of elution buffer. Extracts were stored at −70°C.

PBMCs were probed for *C. pneumoniae* nucleic acid with use of *ompA* gene–specific primers that were developed in-house by Primer Express software and with use of 16S rRNA gene–specific primers [22]. Both primer sets detected 5 isolates of *C. pneumoniae:* one isolated from atheromatous plaques (gift from J. Mahoney), AR39 (gift from C. Wagner), a vascular isolate and a respiratory isolate (gift from M. Maass), and TW-183 (purchased from American Type Culture Collection). The primers detected the target genes in all 5 isolates with a high level of sensitivity (see below).

All primers and fluorescently labeled probes were purchased from Integrated DNA Technology. Syber green was purchased from Molecular Probes. All sample preparation and PCR steps were performed in different hoods in separate rooms with use

of dedicated pipettes. Table 1 shows primer and probe sequences and PCR protocols.

The negative PCR control was PCR water. The positive PCR control was an extract of the isolate of *C. pneumoniae* isolated from an atheroma (gift from J. Mahoney). To control for assay inhibition, nucleic acid extracts were tested by PCR for the gene for human albumin.

The 2 primer sets used were chosen on the basis of results of a Basic Local Alignment Search Tool (BLAST) analysis (National Center for Biotechnology Information) of all published primers at the time of protocol development. To determine the sensitivity of our PCR assays, the 5 aforementioned isolates were diluted, extracted, and studied using both *C. pneumoniae* PCR assays. In addition, 1 mg of *C. pneumoniae* DNA was purchased from Applied Bioscience. PCR for the *ompA* gene detected as few as 10 genomes of organisms and could detect as little as 0.0125 μg of the purchased DNA. PCR for the 16S rRNA gene detected 1 genome of the cultured organisms and as little as 0.00125 pg of the purchased DNA.

The PCR assays were specific for *C. pneumoniae. C. pneumoniae* gene was not detected in uninfected HeLa cells or cultured MOLT cells (precursor of PMBCs). HeLa cells were PCR positive for the human albumin gene. The *C. pneumoniae* isolates were negative for the human albumin gene.

To minimize the risk of false-positive results or contamination issues, PCR for *C. pneumoniae* was performed for replicate aliquots of each extracted sample in separate PCR runs. We required reported results to be reproducible over at least 2 PCR runs. If PCR results for the first 2 aliquots were discordant, a third aliquot from the same sample was run, and the reproducible result was reported. To determine prior exposure to *C. pneumoniae, C. pneumoniae* IgG antibody titers were determined for all patients by microimmunofluorescence [23] in the laboratory of Dr. W. Stamm.

Dichotomous and categorical variables are presented as percentages. The 95% CIs for binomial data were calculated by exact method using the binomial distribution. The χ^2 test was used to compare the dichotomous variables between groups. Statistical analyses were performed using S-PLUS, version 6.1 (Insightful). The authors had full access to the data and take

Table 1. Details of PCR methodology.

Variable, gene target	Sequence or method
Primer sequence	
ompA forward primer	5′-AAG GGC TAT AAA GGC GTT GCT-3′
ompA reverse primer	5′-AGA CTT TGT TCC AGT AGC TGT TGC T-3′
ompA probe	5′-56-FAM/TCC CCT TGC CAA CAG ACG CTG G/3BHQ_1–3′
16S rRNA forward primer	5′-GGT CTC AAC CCC ATC CGT GTC GG-3′
16S rRNA reverse primer	5′-TGC GGA AAG CTG TAT TTC TAC AGT T-3′
16S rRNA probe	5′-56-FAM/ATG CCG CCT GAG GAG TAC ACT CGC AA/3BHQ_1–3
Human albumin forward primer	5′-GCT GTC ATC TCT TGT GGG CTG T-3′
Human albumin reverse primer	5′- AAA CTC ATG GGA GCT GCT GGT T-3′
Human albumin probe	Syber green
Real-time PCR master mix for 10 assays	
ompA gene and human albumin gene	125 μL of TaKaRa 2 × Premix Ex Taq (Takara Biologicals) plus 50 μL of forward primer at 10 μM plus 50 μL of reverse primer at 10 μM plus 50 μL of fluorescently labeled probe at 10 μM plus 110 μL of PCR-grade water; each assay tube contained 24 μL of master mix plus 1 μL of control or unknown
16S rRNA (RT-PCR)	125 μL of Access Quick (Promega) plus 25 μL of forward primer at 10μM plus 25 μL of reverse primer at 10 μM plus 25 μL of fluorescently labeled probe plus 100 μL of PCR-grade water plus avian myeloblastosis virus; each assay tube contained 24 μL of master mix plus 1 μL of control or unknown
PCR protocol on Smart Cycler (Cepheid)	
ompA gene	95°C for 120 s, followed by 40 cycles at 95°C for 15 s and at 60°C for 30 s; optics were on during 60°C step
16S rRNA gene	48°C for 1800 s, followed by 95°C for 120 s, followed by 4 cycles of 94°C for 15 s, 64°C for 30 s, and 72°C for 30 s; followed by a series of 4 cycle steps in which the annealing temperature decreased to 62°C, then 60°C, 59°C, 58°C, 57°C, and 56°C; and finally 21 cycles with the annealing temperature at 55°C; optics were on during the annealing steps
Human albumin gene	Same as for *ompA* with Melt from 60°C to 95°C added at the completion of the PCR protocol

responsibility for its integrity. All authors have read and agree to the article as written.

RESULTS

A total of 86 case patients with CAD and 91 age- and gender-matched control subjects without CAD were enrolled in our study. The age and gender distributions of case patients and control subjects are shown in figure 1. Baseline characteristics of the case and control groups are presented in table 2. Although the control group did not undergo coronary angiography, all control subjects were free of standard cardiac risk factors (table 2). The groups were otherwise similar with regard to factors that were not controlled.

To determine the prevalence of *C. pneumoniae* in PBMCs, PCR was performed on the first blood sample collected from each patient (table 3). None of the case patients or control subjects had a positive *C. pneumoniae* assay result by either *ompA* DNA PCR or 16S rRNA nucleic acid PCR. All samples demonstrated reproducible results in replicate aliquots and were run with appropriate controls. If, as in other studies, a positive result was defined as positivity for only 1 aliquot of a sample, 8 (8.8%; 95% CI, 3.9%–16.6%) of 91 control subjects and 9 (10.5%; 95% CI, 4.9%–18.9%) of 86 case patients would have been classified as positive for *C. pneumoniae.*

All laboratory controls produced expected results in all runs. No inhibition of PCR was found in any sample. A subset of 22 patient samples (from 11 case patients and 11 control subjects) and 8 laboratory controls were sent to the laboratory of Dr. G. Byrne in a blinded manner for PCR analysis; the positive and negative controls yielded appropriate results, and as in our laboratory, all patient samples were uniformly negative.

The majority of patients had evidence of prior exposure to *C. pneumoniae,* as demonstrated by elevated IgG antibody titers. There were no statistically significant differences between the case and control populations with use of the cutoffs for positive results that are commonly found in the literature (table 3).

Forty-two patients, 18 with CAD and 24 healthy control subjects, returned to have all serial samples collected once every 2 months for 8 months during the study period. No *C. pneumoniae* nucleic acid was detected in the PBMCs of any of these samples. These samples were run with the same aforementioned definition of positivity and appropriate controls.

DISCUSSION

None of the patients in our study, either with or without CAD, had evidence of *C. pneumoniae* in PBMCs by PCR. Although control subjects did not have coronary angiography performed, they had no clinical evidence of atherosclerosis and no established risk factors for CAD. Our uniformly negative results include a seasonal analysis with complete data for 5 sampling periods over an 8-month period for 42 patients. Many additional patients returned for >1 visit during the study period to have a blood sample obtained, and none had detectable *C. pneumoniae* nucleic acid in PBMCs (data not shown). As summarized in table 4, our uniformly negative results are in contrast with the wide range of prevalence in 18 similar published controlled studies [4, 24–28].

As indicated in critical reviews, variable PCR results are not a surprise, because of inconsistency of methods (i.e., differences in gene target, amplification procedure, definition of positive results, detection method, and other technical details), as well as problems with contamination [6–8, 31]. These inconsistencies were highlighted in a multicenter comparison trial in which samples were analyzed by 16 test methods at 9 centers by PCR, and positivity ranged from 0% to 60%, with no concordance among laboratories for individual specimens [31]. As recommended [5], we used gene sequence primers (*ompA* and 16S rRNA) that are highly conserved and share no sequences with human genes, and to our knowledge, we were the first to do so. Furthermore, only 3 of the 18 published case-control studies of analysis of PBMCs for *C. pneumoniae* used real-time PCR [27, 29, 30], as opposed to other PCR methods (e.g., nested

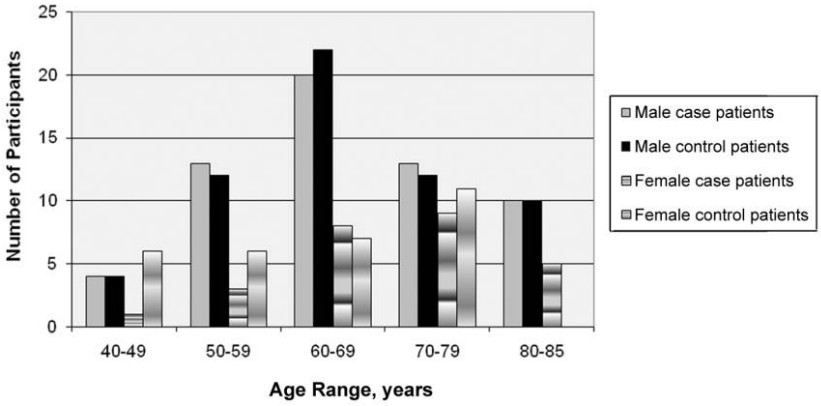

Figure 1. Age and gender distribution of case patients and control subjects

Table 2. Characteristics of the study population.

Characteristic	No. (%) of patients	
	Case patients with coronary artery disease (n = 86)	Control subjects (n = 91)
Percutaneous coronary intervention	48 (56)	0 (0)
Coronary artery bypass graft surgery	30 (35)	0 (0)
Coronary artery disease risk factors		
Hypertension	61 (71)	0 (0)
Hypercholesterolemia	68 (79)	0 (0)
Diabetes mellitus	21 (24)	0 (0)
Tobacco use		
Current	6 (7)	0 (0)
Former (<10 years prior to enrollment)	6 (7)	0 (0)
Ever	41 (48)	37 (41)
Peripheral vascular disease	8 (9)	0 (0)
Cerebrovascular disease	9 (10)	0 (0)
Chronic obstructive pulmonary disease	17 (20)	0 (0)
Asthma	7 (8)	9 (10)
Cancer	8 (9)	7 (8)
Renal failure	3 (3)	0 (0)
Family history of heart disease	25 (29)	11 (12)
Current alcohol use	39 (45)	65 (71)

PCR), which are now considered to be less specific. Nested PCR, used in most of the 18 studies, has since been shown to be unreliable in detecting *C. pneumoniae* in atheromas secondary to contamination through amplicon carry-over, causing 8% of negative controls to yield positive results in 1 analysis [32]. It is likely that this methodological flaw occurs in studies of PBMCs. Of interest, the 3 studies that used real-time PCR reported a prevalence of <10% among control subjects and case patients (table 4). Furthermore, 1 of these 18 case-control studies demonstrated no detectable *C. pneumoniae* DNA in PBMCs by PCR [27], similar to our findings. In that negative study [27], case patients with carotid atherosclerotic plaques were compared with control subjects with use of real-time PCR, similar to our study, and none of the patients had a positive PCR result, which further supports the hypothesis that real-time PCR may be a more sensitive, specific, and reliable methodology than other PCR assays [27].

Another important difference in our methods was the strict definition of both case and control populations. Case patients had definite CAD determined on the basis of angiography within the 18 months prior to enrollment. Control subjects had no history of or risk factors for CAD. Although use of such selection criteria introduces some bias, as discussed below, it also ensures that 2 very distinct groups were studied. A negative association of *C. pneumoniae* in PBMCs and such different risk groups is important.

An additional substantive difference in methods is the definition of a positive PCR result. We required reproducibility in replicate aliquots. The definition of a positive PCR result is not provided in 8 of the 18 published controlled studies [4, 24, 26, 28]. The other 10 studies used highly variable definitions of a positive result [4, 25, 27]; it appears that any single determination of positivity was considered to be a positive result in most of these studies [4, 25]. Of interest, if we had accepted a positive result in only 1 of the replicate aliquots, then the prevalence of positivity among case patients and control subjects would have been 8.8% and 10.5%, respectively. These rates suggest that other factors besides true positivity, such as technical factors, can influence a single run if not repeated for validity.

The validity of our results is further supported by the use of multiple appropriate positive and negative laboratory controls, which produced expected results in each run. In addition, we exchanged patient specimens and positive and negative control specimens with another laboratory, and their results were identical to ours. Therefore, it seems unlikely that any technical reason would explain our negative results.

We used a standard *C. pneumoniae* microimmunofluorescent antibody assay to document prior exposure of our study population to *C. pneumoniae* (table 3) [5, 18]. Of interest, we found no difference in the prevalence of IgG antibody titers between the group with CAD and the group without CAD. This finding

Table 3. Comparison of results of PCR for *Chlamydophila pneumoniae* nucleic acid extracted from PBMCs from case patients and control subjects.

Test	Patients with coronary artery disease (n = 86)	Control subjects (n = 91)
Prevalence of positivity, % (95% CI)		
PCR for *ompA* gene	0 (0–4.2)	0 (0–4.0)
PCR for 16s rRNA	0 (0–4.2)	0 (0–4.0)
C. pneumoniae IgG antibody titer, no. (%) of patients		
⩾1:16[a]	64 (74)	67 (74)
⩾1:32[a]	58 (67)	61 (67)
⩾1:64[a]	56 (65)	55 (60)

NOTE. Results are for the first blood sample obtained from each participant. Also provided are the results of the microimmunofluorescent antibody assay.

[a] $P > .5$, by χ^2 test.

is in contrast with older literature, which suggests that antibody is found more often in patients with CAD, but is in concordance with more-recent reports [4].

The disparity of findings in the literature, coupled with our negative results, suggests that the detection of *C. pneumoniae* in PBMCs is not a reliable marker for the presence of *C. pneumoniae* in atherosclerotic vascular lesions. Of interest, even after disruption of plaque with angioplasty of atheromatous vessels, a procedure expected to release organisms into the bloodstream, Smieja et al. [18] found no increase in the prevalence of *C. pneumoniae* positivity with use of nested PCR methods. Our results do not invalidate other data demonstrating *C. pneumoniae* nucleic acid within atheromas. Even if *C. pneumoniae* exists in atheromas of selected patients, our negative results provide evidence against movement of *C. pneumoniae*–infected cells in and out of atheromas that can be detected in circulating PBMCs. Similarly, our data do not preclude *C. pneumoniae* circulating outside cells.

We did not demonstrate a seasonal variation in results, which suggests that the seasonal effect considered in other studies [16–21] may be more related to an increase in the rate of seasonal respiratory tract infection during the winter months than to atheroma infection. *C. pneumoniae* may be intermittently shed from atheromatous plaques and, therefore, only periodically detected in PBMCs [20, 21]. If this is the case, our results suggest that obtaining samples every 2 months is not adequate to find *C. pneumoniae* in PBMCs.

There are several limitations to our study, including those inherent to all case-control studies. The statistical power of the study is limited by the small number of participants; however, because the results were uniformly negative, this limitation is not significant.

Another limitation is that the 2 study groups were very different with regard to cardiac risk factors. It is challenging to define a control group that does not have a disease such as CAD. Many case-control studies have used normal angiogram

Table 4. Comparison of our results with those of 18 published studies attempting to detect *Chlamydophila pneumoniae* DNA in PBMCs by PCR.

Variable	Case patients	Control subjects
18 Published studies [4,24–28]		
No. of patients, range	18–913	19–292
Prevalence of PCR positivity, % range	0–72	0–50
Combined total positive results	387	219
Combined total no. of patients	2295	1594
Combined total prevalence of PCR positivity, %	17	14
Our study		
No. of patients	86	91
Prevalence of PCR positivity, %	0	0
3 Studies that used real-time PCR [27, 29, 30]		
No. of patients	45, 149, 57	30, 120, 38
Prevalence of real-time PCR positivity, %	4, 9, 0	3, 7, 0

findings to define their control group, but up to 20% of patients, usually women, with "normal" angiogram findings may have diffuse small vessel atherosclerotic disease [33]. Given the disparity in the literature, we chose 2 groups that were different enough with regard to cardiac risks that, if there is indeed an association between *C. pneumoniae* nucleic acid in PBMCs and atherosclerotic disease, we should have demonstrated a difference in this nucleic acid detection in our 2 groups.

In conclusion, our results do not support the majority of the earlier literature describing a substantive prevalence of *C. pneumoniae* genome in circulating PBMCs. Our results do not preclude the possibility of the presence of "persistent" *C. pneumoniae* in the lung or in atherosclerotic plaques. If the latter is true, our results suggest that testing for *C. pneumoniae* nucleic acid in circulating PBMCs is not useful as a surrogate marker of infection of atherosclerotic plaques.

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Potential conflicts of interest. D.N.G. is a consultant for Pfizer, Schering-Plough, Bayer, Advanced Life Sciences, and Pacific Beach Biosciences, and is on the speakers' bureau for Merck. All other authors: no conflicts.

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