Is *Chlamydia pneumoniae* Infection Associated With Stroke in Children With Sickle Cell Disease?

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ABSTRACT. *Background.* **Stroke is often a devastating complication of sickle cell disease (SCD). Most children with SCD-related stroke have stenotic and occlusive disease of cerebral blood vessels due to intimal hyperplasia. This hyperplasia is hypothesized to result from an inflammatory response similar to that in atherosclerosis and has been attributed to infection by *Chlamydia pneumoniae*.**

Objective. **To determine whether *C pneumoniae* infection is associated with stroke and cerebrovascular disease, including transient ischemic attacks and abnormal transcranial Doppler examinations, in children with SCD.**

Methods. **Children with SCD on chronic transfusion due to a history of stroke, transient ischemic attack, or abnormal transcranial Doppler; children with SCD without stroke; healthy controls; and children being transfused for other reasons were enrolled. Peripheral blood and nasopharyngeal (NP) swab specimens were collected from all patients. In patients on transfusion, pretransfusion specimens and samples from the unit of packed red blood cells being transfused were obtained. Peripheral blood monocytic cells (PBMCs) and NP swab specimens were cultured for *C pneumoniae* in HEp-2 cells. *C pneumoniae* polymerase chain reaction was performed on PBMCs with a nested touch-down method with primers from the *omp-1* gene (in duplicate) and a second real-time polymerase chain reaction by using 16S ribosomal RNA primers.**

Results. ***C pneumoniae* DNA was detected in the PBMCs of 1 of 14 (7.1%) children with SCD on chronic transfusion, 1 of 10 (10%) sickle cell controls, 1 of 10 (10%) healthy controls, and none of the 5 children receiving chronic transfusion for other reasons. It was not detected in specimens from transfusion units. One child with SCD and stroke, 1 sickle cell control, and 1 transfusion control had positive NP cultures for *C pneumoniae*. *C pneumoniae* DNA was not detected in their PBMCs, and all 3 children were asymptomatic. *C pneumoniae* was not detected by culture of PBMCs from any of the patients after 7 passages.**

Conclusion. **Stroke in children with SCD does not seem to be associated with C *pneumoniae* infection in our population.** *Pediatrics* **2004;113:e318–e321. URL: http://www.pediatrics.org/cgi/content/full/113/4/e318; *peripheral blood monocytes, transfusion, cerebrovascular disease.***

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ABBREVIATIONS. SCD, sickle cell disease; PBMC, peripheral blood monocytic cell; PCR, polymerase chain reaction; NP, nasopharyngeal; LC, Lightcycler; IFU, inclusion-forming unit(s).

Stroke is often a devastating complication that affects 10% of children with sickle cell disease (SCD).[1] Most children with sickle cell-related stroke have stenotic and occlusive lesions of large and middle caliber cerebral blood vessels due to intimal hyperplasia.[2] This hyperplasia is hypothesized to result from an inflammatory response due to abnormal adhesion of sickle cells to vascular endothelium and is similar to that seen in atherosclerosis.[2,3] For atherosclerosis, the exact cause of this inflammation is unknown, but chronic infection has been suggested, with *Chlamydia pneumoniae* being a likely potential pathogen.[3] One of the major limitations of studies of the association of *C pneumoniae* and atherosclerosis has been the lack of a sensitive and specific test to determine who is actually infected.[3] The presence of *C pneumoniae* DNA in circulating peripheral blood monocytic cells (PBMCs) has been proposed as an indicator of active intravascular infection. *C pneumoniae* DNA was detected by polymerase chain reaction (PCR) in PBMCs in 0% to 59% of adult patients with coronary artery disease.[3,4] However, *C pneumoniae* DNA was also detected in the PBMCs of 0% to 46% of normal blood donors.[3,4]

C pneumoniae is a common cause of respiratory infection in adults and children and a frequent cause of acute chest syndrome in children with SCD.[5,6] Chronic respiratory infection is known to occur.[5] Styles et al[7] reported in a preliminary study an association of *C pneumoniae* infection with sickle cell-related stroke. *C pneumoniae* DNA was detected in PBMCs of 5 of 10 (50%) children with SCD receiving chronic transfusion for stroke, compared with none of 10 children with SCD and no stroke.

The objective of the present study was to determine whether there indeed is an association of *C pneumoniae* infection with sickle cell-related stroke. In that the usual therapy for either secondary[8] or primary[9] stroke prevention in children with SCD is chronic blood transfusion, we also examined whether the presence of *C pneumoniae* DNA in chil-

dren with SCD and stroke could be an artifact of transfusion.

METHODS

Patients

Children with SCD with a history of stroke, transient ischemic attacks, or abnormal transcranial Doppler examination[9] on chronic transfusion; children with SCD without a history of stroke; patients transfused for other reasons; and hematologically normal children at health maintenance visits were invited to participate in the study. This study was approved by the State University of New York Downstate Medical Center Institutional Review Board. Informed consent was obtained from the parent or legal guardian of each child, and assent was obtained from all children 7 to 18 years old.

Specimen Collection

Nasopharyngeal (NP) specimens for *C pneumoniae* culture were obtained by using aluminum-shafted Dacron swabs, which were passed through the nares to the posterior nasopharynx. The swabs then were placed into transport media as described.[10]

Preparation of PBMCs

Peripheral blood was collected by venipuncture for PBMCs by using 8-mL CPT tubes containing sodium citrate (Becton Dickinson, Franklin Lakes, NJ). Blood specimens were stored at room temperature until processing, centrifuged within 2 hours, and processed according to the manufacturer's package insert. The processed cells were frozen at −70°C until ready for culture and DNA-extraction procedures.

Culture of *C pneumoniae*

PBMCs and NP specimens were cultured for *C pneumoniae* in HEp-2 cells grown in 96-well microtiter plates as described.[10] Cultures of PBMCs were passaged 7 times.

C pneumoniae PCR

DNA was extracted from 200 μL of processed PBMCs by using the MagnaPur Lightcycler (LC) Robot (Roche Molecular Systems, Pleasanton, CA). Detection of *C pneumoniae* DNA in PBMCs was performed by using 2 different PCR methods: a nested touchdown PCR with primers from the *omp-1* gene (in duplicate)[11] and a real-time PCR.[12] Real-time PCR was performed by using the existing primer set CPN90 and CPN91; a TaqMan probe was designed for use with CPN90 and CPN91 and the Roche Molecular Systems LC. Primer and probe sequences for the *C pneumoniae* CPN-LC assay were primer CPN90 (5′-TCTCAACCCCATCCGTGTCGG-3′), primer CPN91 (5′-GCGGAAAGCTGTATTTCTACAGTT-3′), and probe CPNTM (5′-6FAM-ATGCCGCCTGAGGAGTACACTCGCAA-3′). PBMC samples for real-time PCR testing were analyzed under the following conditions: 15 μL of master mix and 5 μL of template DNA with a primer and probe concentration of 10 pm per PCR and a $MgCl_2$ concentration of 4 mM per PCR. The $MgCl_2$, PCR-grade water, and Faststart enzyme were provided by the Roche Molecular Systems Faststart Hybridization Probe Kit. The amount of enzyme (2 μL) was based on manufacturer recommendations. DNA extraction and PCR setup and amplification were all performed in separate rooms. Aerosol barrier-resistant pipette tips were used for all steps in the PCR setup. Cycling conditions were 95°C for 30 seconds of preincubation followed by 45 cycles at 95°C for 0 seconds, 65°C for 10 seconds, and 72°C for 5 seconds, followed by 3 minutes of cooling at 40°C at the end of the cycling protocol. Data collection was performed during extension by the LC software, which calculated cutoff values for positives based on a positive control panel. Negative and positive controls were included in both the extraction and PCR phase of testing. The positive controls consisted of a panel of at least 4 different concentrations of *C pneumoniae*: 400 inclusion-forming units (IFUs)/PCR, 40 IFU/PCR, 4 IFU/PCR, and 0.4 IFU/PCR.

RESULTS

A total of 39 patients were enrolled, including 13 of 20 children at Downstate Medical Center with sickle cell-related cerebrovascular disease currently on transfusion therapy (7 who had strokes, 2 with recurrent transient ischemic attacks, and 4 with abnormal transcranial Doppler examinations); these children were classified as sickle cell stroke for purposes of analysis. Ten sickle cell control children without a history of stroke, 10 healthy control children, and 5 children receiving chronic transfusion for other reasons (Diamond-Blackfan anemia and thalassemia major) were also enrolled. As shown in Table 1, ages ranged from 7 to 19 years, and 14 patients (35.9%) were male. The mean duration of transfusion therapy in the children with SCD and stroke was 28.22 months (range: 1–72 months), and the median duration was 26 months at the time of study entry.

C pneumoniae DNA was detected in PBMCs of 1 of 14 (7.1%) children with SCD and stroke and 1 of 10 (10%) non–stroke sickle cell controls, both by only 1 of the 3 replicate PCR tests used (Table 2). *C pneumoniae* DNA was detected in the PBMCs of 1 of 10 (10%) healthy control children, in this case in all 3 PCR replicates. The prevalence of *C pneumoniae* DNA was not significantly different between the 3 groups ($\chi^2 = 0.1752$ with Yates continuity correction; $P > .9$). *C pneumoniae* DNA was not detected in the PBMCs of any of the 5 transfusion controls or in specimens from the transfusion units. *C pneumoniae* was not detected by culture in any subject's PBMCs. A total of 3 children had positive NP cultures for *C pneumoniae*: 1 with SCD and stroke, 1 with SCD without stroke, and 1 transfusion control. *C pneumoniae* DNA was not detected in the PBMCs of any of these children, all of whom were asymptomatic.

DISCUSSION

Cerebrovascular accident is a common complication of SCD, occurring at a rate of ~0.5 events per 100 patient-years of follow-up.[1] Of children with sickle cell-related stroke, 70% to 90% have stenotic and occlusive changes in the large- and middle-caliber cerebral blood vessels;[2] this vasculopathy has been attributed to damage caused by abnormal adherence of sickle cells[13] and leukocytes[14] to vascular endothelium, resulting in intimal hyperplasia and narrowing of the vessel lumen.[15] Vascular disease is often extensive,[16] and stroke recurs in 47% to 90% of untreated children who have an initial stroke.[17–19] This risk is reduced greatly by implementation of a chronic transfusion program.[8]

In the non–sickle cell adult population, similar vascular abnormalities are seen in association with atherosclerosis, and there is considerable interest and controversy as to the role of infection, specifically the role of *C pneumoniae* infection, in the pathogenesis of these lesions. The rates of identification of *C pneu-*

TABLE 1. Demographic Characteristics of Population

Patient Group	*N*	Mean Age, y (range)	% Male
SCD with stroke	14	12.2 ± 3.4 (7–14.5)	35.7
SCD controls	10	13.4 ± 3.7 (7–19)	20
Healthy controls	10	12.6 ± 3.5 (8–18)	50
Transfusion controls	5	14.9 ± 4.4 (7.5–18.5)	40

TABLE 2. Results of *C pneumoniae* Cultures and PCR

Patient No.	Group	Age, y	Gender	NP Culture	Monocyte Culture	PCR 1	PCR 2	LC PCR
1	HC	10	M	−	−	−	−	−
2	HC	8	F	−	−	+	+	+
3	HC	8	F	−	−	−	−	−
4	HC	16	M	−	−	−	−	−
5	HC	15	F	−	−	−	−	−
6	HC	11	M	−	−	−	−	−
7	HC	11	M	−	−	−	−	−
8	HC	16	M	−	−	−	−	−
9	HC	18	F	−	−	−	−	−
10	HC	13.5	F	−	−	−	−	−
11	SCC	7	F	−	−	−	−	−
12	SCC	18	F	−	−	−	−	−
13	SCC	14	F	−	−	−	−	−
14	SCC	16	M	−	−	−	−	−
15	SCC	13	F	−	−	−	−	−
16	SCC	19	F	−	−	−	+	−
17	SCC	13	F	−	−	−	−	−
18	SCC	9	F	+	−	−	−	−
19	SCC	13	F	−	ND	−	−	−
20	SCC	11	M	−	−	−	−	−
21	SCS	13	F	+	−	−	−	−
22	SCS	16	M	−	−	−	−	−
23	SCS	14	M	−	−	−	−	−
24	SCS	14	F	−	−	−	−	+
25	SCS	9.5	F	−	−	−	−	−
26	SCS	8	F	−	−	−	−	−
27	SCS	9.5	M	−	−	−	−	−
28	SCS	14.5	F	−	−	−	−	−
29	SCS	16	F	−	−	−	−	−
30	SCS	11	F	−	−	−	−	−
31	SCS	11	F	−	−	−	−	−
32	SCS	9	M	−	−	−	−	−
33	SCS	18	F	−	−	−	−	−
34	SCS	7	M	−	−	−	−	−
35	TC	18	M	−	−	−	−	−
36	TC	14.5	F	−	−	−	−	−
37	TC	7.5	F	−	−	−	−	−
38	TC	18.5	F	−	−	−	−	−
39	TC	16	M	+	−	−	−	−

HC indicates healthy control; M, male; F, female; SCC, sickle cell control; SCS, sickle cell stroke; TC, transfusion control; ND, not done.

moniae in human atheromas, predominantly by PCR, range from 0% to 100%,[3] although the organism has actually been isolated from atheroma tissue in <10% of specimens when culture was performed.[3] Inconsistency of results is due, in part, to the lack of standardized methods, especially those using PCR.[3,20] Recent studies document major problems with both inter- and intralaboratory reproducibility of in-house PCR assays for *C pneumoniae*.[3,20]

It has been suggested that the presence of *C pneumoniae* in PBMCs may act as a surrogate marker for infection with *C pneumoniae* in adults with cardiovascular disease.[3,4] This is based on the hypothesis that the organism gains access to the vascular system from a respiratory focus through infection of tissue or alveolar macrophages. *C pneumoniae* has been identified in buffy coats and PBMCs of mice after intranasal inoculation by culture and PCR.[21] Similar to the experience with atheroma tissue, the reported prevalence of *C pneumoniae* DNA in PBMCs has varied significantly, ranging from 0% to 59% of patients with coronary artery disease and from 0% to 46% of healthy blood donors.[3,4] Data on the association of the presence of *C pneumoniae* DNA in PBMCs and atheroma tissue from patients with coronary artery disease in individual studies also have been inconsistent.[3] Some of this variation is probably due to variations in PCR methods used, as discussed above. There have been no published studies of the prevalence of *C pneumoniae* DNA in PBMCs of children. It is also not known whether carriage of *C pneumoniae* DNA in circulating white blood cells represents a viable infection; actual isolation of viable *C pneumoniae* from PBMCs by culture is rare.[3]

Given these methodologic concerns, we felt compelled to attempt to confirm the results reported by Styles et al.[7] The results of the present study are strikingly different, which may be due to methodologic issues discussed above. We do not have details of the PCR methods used by Styles et al,[7] and they did not perform NP cultures or study a healthy control group or children on chronic transfusion for other reasons.

Our results show no association of infection by *C pneumoniae* with stroke in children with SCD. There was also no association of presence of *C pneumoniae* DNA in PBMCs with either NP infection or chronic transfusion. The prevalence of *C pneumoniae* DNA in these children (7.1%–10%) was similar to that reported in healthy adult blood donors.[3,5] In 2 of the children in whom *C pneumoniae* DNA was detected in PBMCs, only 1 of 3 PCR tests were positive, sug-

gesting a low copy number. Tondella et al[22] reported similar results in an adult population. They analyzed 228 PBMC specimens by 4 PCR assays including 2 real-time assays (1 of which was used in the present study), 1 nested PCR assay, and the nested touchdown assay (also used in the present study). One of the real-time assays and the nested PCR assay each detected *C pneumoniae* DNA in a single but different PBMC specimen, consistent with low amounts of *C pneumoniae* DNA.

The overall prevalence of asymptomatic *C pneumoniae* NP infection (7.7%) in the present study was similar to that reported previously in children and adults.[23] We conclude that *C pneumoniae* is not involved in sickle cell-associated cerebrovascular disease.

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