Aquaporin 5 Gene Promoter −1364A/C Polymorphism Associated with 30-day Survival in Severe Sepsis

Michael Adamzik, M.D.,* Ulrich H. Frey, M.D.,† Stephan Möhlenkamp, M.D.,‡ André Scherag, Ph.D.,§ Christian Waydhas, M.D.,‖ Günther Marggraf, M.D.,# Marc Dammann, M.D.,** Jörg Steinmann, M.D.,†† Winfried Siffert, M.D.,‡‡ Jürgen Peters, M.D.§§

ABSTRACT

Background: Because the aquaporin (AQP) 5 promoter −1364A/C polymorphism is associated with altered AQP5 expression, this association could have an impact on key mechanisms in sepsis, such as cell migration, activity of the renninangiotensin- aldosterone system (RAAS), and water transport across biologic membranes. Therefore, we tested the hypothesis that the AQP5 promoter −1364A/C polymorphism is associated with increased 30-day survival in severe sepsis.

Methods: In a prospective study, adults with severe sepsis (N = 154) were genotyped for the AQP5 promoter −1364A/C polymorphism. The clinical endpoint was 30-day survival. Kaplan–Meier plots and multivariate proportional hazard analyses were used to evaluate the relationship between genotypes and clinical outcomes.

Results: Thirty-day survival was significantly associated with AQP5 −1364A/C genotypes ($P = 0.001$). Survival rates were 57% for AA genotypes (n = 90) but 83% for combined AC/CC genotypes (56 *vs.* 8, respectively). Multivariate proportional hazard analysis including sex, age, Simplified Acute Physiology Score II, Sequential Organ Failure Assessment score, body mass index, necessity for continuous hemofiltration/dialysis, concentrations of plasma angiotensin II, serum aldosterone, C-reactive protein, and interleukin 6 as covariates revealed the AQP5 −1364A/C polymorphism as a strong and independent prognostic factor for 30-day survival. In this analysis, homozygous AA subjects were at high risk for death within 30 days (hazard ratio, 3.59; 95% CI, 1.47–8.80; $P = 0.005$) compared with AC/CC genotypes.

Conclusion: The C-allele of the AQP5 −1364A/C polymorphism is associated with increased 30-day survival in patients with severe sepsis. This finding suggests the importance of variations in expression of AQP5 channels in severe sepsis.

What We Already Know about This Topic

- Aquaporin 5 plays key roles in homeostasis and response to sepsis. Polymorphism of the promoter for this molecule might account for the wide variability in mortality from sepsis.

What This Article Tells Us That Is New

- In 154 patients with sepsis, polymorphism in the promoter for Aquaporin 5 was clearly associated with mortality risk, with a nearly four-fold difference among genotypes.

ALTHOUGH wide variability exists regarding patient outcomes in severe sepsis, some of this variability may be caused by genetic variation. A potential candidate for investigation is the gene-encoding aquaporin (AQP) 5,[1] which mediates key mechanisms of inflammation that prevail in sepsis, including cell migration and proliferation,[2] activity of the reninangiotensin-aldosterone system (RAAS),[3] and the transport of water across biologic membranes.[4]

On sequencing the AQP5 promoter region among healthy white patients (N = 50), we previously described[3] a novel, functional, and common single nucleotide (−1364A/C) polymorphism in the AQP5 gene promoter:

> Substitution of C for A at position −1364 was associated with increased binding of transcription factors, as shown for nuclear extracts from HeLa cells, but reduced transcriptional activation of the AQP5 gene in response to serum and cyclic adenosine monophosphate. This latter finding appears of special interest since stimulation of the vasopressin V2-receptor by arginine-vasopressin results in increased AQP5 messenger RNA concentrations and increased translocation of AQP5 to the plasma membrane which is mediated *via* an increase in cyclic adenosine monophosphate and subsequent

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* Lecturer, Physician, † Physician, §§ Professor, Physician, Klinik für Anästhesiologie und Intensivmedizin, ‡ Lecturer, Physician, Klinik für Innere Medizin, § Doctor of Human Biology Institut für Medizinische Informatik, Biometrie und Epidemiologie, ‖ Professor, Physician, Klinik für Unfallchirurgie, # Professor, Physician, Klinik für Thorax- und Kardiovaskuläre Chirurgie, ** Physician, Klinik für Allgemein-, Viszeral- und Transplantationschirurgie, †† Physician, Institut für Mikrobiologie, ‡‡ Professor, Physician, Institut für Pharmakogenetik, Universität Duisburg-Essen, Universitätsklinikum Essen, Germany.

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Address correspondence to Dr. Adamzik: Klinik für Anästhesiologie und Intensivmedizin, Universitätsklinikum Essen, Hufelandstr. 55, D-45122 Essen, Germany. michael.adamzik@uk-essen.de. This article may be accessed for personal use at no charge through the Journal Web site, www.anesthesiology.org.

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Table 1. Baseline Characteristics of Patients with Severe Sepsis as Grouped by AQP5 −1364A/C Genotype (N = 154)

Characteristic	AA (n = 90)	AC/CC (n = 64)	*P* Value
Demographic data			
Age, yr	56 ± 15	60 ± 16	0.14
Sex, men/women, No.	58/32	40/24	0.87
Body mass index, kg/m^2	27 ± 6	27 ± 4	0.99
Medical history, No.	—	—	—
Cardiovascular disease	18	13	NA
Hemato-oncology disease	4	3	
Gastrointestinal disease	28	16	
Gastrointestinal cancer	11	7	
Lung disease	14	9	
Lung cancer	2	2	
Urogenital disease	5	4	
Urogenital cancer	3	3	
Skin and fascial infection	3	2	
Trauma	2	1	
Continuous hemofiltration/dialysis, %*	43	39	0.14
Mechanical ventilation, %*	100	100	1.00
Plasma angiotensin II (Q1, Q2, Q3), pg/ml†	(10, 16, 28)		0.13
Serum aldosterone (Q1, Q2, Q3), ng/l†	(12, 45, 106)		0.61
C-reactive protein, mg/dl	13 ± 9	15 ± 10	0.23
IL-6 (Q1, Q2, Q3), pg/ml†	(40, 95, 450)		0.54
Procalcitonin, ng/ml	22 ± 55	14 ± 31	0.34
SAPS II	49 ± 15	49 ± 17	0.90
SOFA score	12 ± 4	12 ± 6	0.39
Gram positive isolates only, %	42	39	NA
Gram negative isolates only, %	35	31	
Mixed bacterial isolates, %	9	15	
Viral isolates, %	0	0	
Fungal isolates, %	1	2	
Negative cultures, %	13	13	

All data are presented as mean ± SD (Welch *t* test) unless otherwise specified.

* *P* values based on Fisher exact test; † Quartiles and *P* values based on exact Wilcoxon Mann–Whitney U test.

IL-6 = interleukin 6; NA = not available (no statistical test applied); SAPS II = Simplified Acute Physiology Score II; SOFA = Sequential Organ Failure Assessment score.

activation of the protein kinase A pathway.[4–6] Thus, the C-allele may facilitate the binding of an inhibitory transcription factor. These results were corroborated by findings showing that the C-allele was associated with reduced AQP5 messenger RNA transcripts in human right atrial specimens and reduced AQP5 protein expression in red cell membranes.[3]

Furthermore, we found that the C-allele is associated with suppression of the RAAS in response to a high salt diet. In addition, RAAS suppression in individuals carrying the AA genotype was significantly blunted.[3]

Thus, there are many reasons to suspect that AQP5 promoter polymorphism could affect key mechanisms in sepsis and influence survival. Accordingly, we prospectively tested the hypothesis that the −1364A/C AQP5 promoter polymorphism is associated with increased 30-day survival in severe sepsis.

Materials and Methods

Patients

This study was reviewed and approved by the Ethics Committee at the University Hospital of Essen (Nordrhein-Westfalen, Germany). Informed consent was obtained from all patients or from patient guardians, as appropriate. Using the criteria for severe sepsis defined by Bone *et al.*,[7] 154 patients (90 men, 64 women; mean ± SD age, 57 ± 16 yr) admitted to the intensive care unit at the University Hospital of Essen were considered eligible for the study during the 2-yr study period. All patients were white Germans of Caucasian ethnicity. Clinical and demographic data on study entry, including Simplified Acute Physiology Score II[8] and Sequential Organ Failure Assessment score,[9] were gathered during the first 24 h after severe sepsis criteria were met (table 1). All patients were observed for 30-day survival as calculated from primary diagnosis of severe sepsis. Patients were treated using a multimodal model that included analgosedation, fluid administration, and protective mechanical ventilation as well as hemodynamic, antibiotic, and diagnostic management. Continuous hemofiltration/dialysis, as required, was technically performed by the hospital's Nephrology department, according to standardized protocols.

Aldosterone and Angiotensin II Concentration Measurements. Blood for determination of plasma angiotensin II

and serum aldosterone concentrations was collected from patients within 24 h of meeting severe sepsis criteria. For plasma angiotensin II measurements, blood was collected into prechilled 10-ml syringes, prepared with 125 mmol EDTA and 26 mmol phenanthroline (Merck KGaA, Darmstadt, Germany) to inhibit angiotensin-converting enzyme. Samples were centrifuged (10 min at 4°C) immediately after collection, rapidly stored at −21°C after centrifugation, and subsequently analyzed. Plasma samples were extracted, and, after sample purification, immunoreactive angiotensin II was measured in duplicate by radioimmunoassay, as previously described.[10] The coefficient of variation was 5%. Serum aldosterone was measured by a commercially available radioimmunoassay kit (Aldosterone Maia, Serono, Freiburg, Germany), as previously described.[11]

DNA Genotyping. DNA was extracted from whole blood using the QIAamp® kit (QIAGEN, Hilden, Germany). For genotyping the −1364A/C polymorphism by Pyrosequencing®, polymerase chain reaction was performed with the forward AQP5-SE 5′-GAAACTGCAGGATGAGAGAAAT-3′, and the biotinylated reverse AQP5-AS 5′-TCTCTGTTCTCCACCTCTCCA-3′ resulting in a 120-nt fragment. After denaturation at 94°C, 40 cycles of DNA amplification were done using Taq polymerase chain reaction Eppendorf® MasterMix (Eppendorf AG, Hamburg, Germany) for 40 s at 94°C, 40 s at 53°C, and 40 s at 72°C. The biotinylated strand was captured on streptavidin Sepharose beads and annealed with AQP5-Seq 5′-CAGAGAGACTAAGACAGCA-3′. Pyrosequencing was performed using PSQ HS 96 Gold Reagent Kits (Biotage, Uppsala, Sweden).

Statistical Analysis

The AQP5 −1364A/C genotype distributions were tested for deviations from Hardy-Weinberg equilibrium (exact two-sided *P* value 1.00). Explorative comparisons by AQP5 −1364A/C genotypes (AC/CC *vs.* AA) were performed for several clinical patient characteristics (table 1). AC and CC were summarized because of the low frequency (5.2%) of the CC genotype.

The clinical endpoint was 30-day survival dependent on AQP5 −1364A/C genotype. Survival probabilities were graphically assessed by the Kaplan–Meier method. The log-rank test was used to evaluate the univariate relationship between AQP5 −1364A/C genotype and clinical outcomes. Afterward, we performed multivariate Cox regression analyses assessing the joint impact of AQP5 −1364A/C genotype as well as patient sex, age, Simplified Acute Physiology Score II, Sequential Organ Failure Assessment score, body mass index, hemofiltration/dialysis status, and concentrations of plasma angiotensin II, serum aldosterone, C-reactive protein, and interleukin 6 as predictors for the clinical outcome (*i.e.*, 30-day survival). Model diagnostic of the proportional hazards assumption for the AQP5 −1364A/C genotype comprised graphical and formal investigations—none of which indicated strong evidence for a deviation from the proportional-hazards assumption. Multivariate analyses included two steps with a focus on AQP5 −1364A/C genotype and 30-day survival. In the initial model, all main effects were investigated simultaneously (table 2). To avoid overfitting, a restricted model with only five variables was assessed afterward using only those predictors with a *P* value of 0.05 or lower in either the univariate or initial multivariate comparison.

CIs were calculated with coverage of 95%. All reported *P* values are nominal and two-sided with an α significance level of 5%.

Results

Thirty-day survival was significantly associated with AQP5 −1364A/C genotypes ($P = 0.001$, fig. 1). Thirty-day survival rates were 57% (39 of 51) for AA genotypes (n = 90) and 83% (11 of 53) for AC/CC (56 and 8, respectively) genotypes.

Multivariate Cox regression analyses revealed AQP5 −1364A/C genotype status as both a strong (estimated effect size) as well as an independent prognostic factor when jointly considering other predictors of 30-day survival outcomes.

Homozygous AA subjects had a high and approximately four-fold greater risk of death (hazard ratio, 3.59; 95% CI, 1.47–8.80; $P = 0.005$) compared with AC/CC genotypes (table 2). Required continuous hemofiltration/dialysis (hazard ratio, 2.76; 95% CI, 119–6.40; $P = 0.018$) was the second best independent, but weaker, prognostic factor for 30-day survival in the restricted multivariate model. Finally, interleukin 6 revealed some evidence for independent prognostic information but did not meet the 5% significance level (hazard ratio per $\log_{10}$, 1.42 pg/ml; 95% CI, 0.92–2.18; $P = 0.114$). Plasma angiotensin II concentration was not associated with 30-day survival (table 2 and fig. 2).

Genotype frequencies and patient clinical characteristics as grouped by genotype are displayed in table 1. We found no evidence for significant associations of AQP5 −1364A/C genotypes with plasma angiotensin II and serum aldosterone concentrations, sex, age, Simplified Acute Physiology Score II, Sequential Organ Failure Assessment score, body mass index, or necessity for continuous hemofiltration/dialysis. Moreover, descriptively there was also no genotype-dependent pattern for infection type or primary diagnosis at hospital admission.

Discussion

This study shows for the first time that the AA genotypes of the AQP5 −1364A/C polymorphism are associated with significantly and substantially increased 30-day mortality in patients with severe sepsis. Furthermore, AQP5 −1364A/C polymorphism represents an independent—and the most important—prognostic factor for survival in our patient sample. The estimated hazard ratio of nearly 4 for the AC/CC genotypes compared with the homozygous AA genotype not only suggests that the C-allele of the AQP5

Table 2. Cox Regression Analyses in Patients with Severe Sepsis (N = 154)

	Univariate		Multivariate			
			Initial		Restricted	
Covariable	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value	Hazard Ratio (95% CI)	*P* Value
Aquaporin 5 −1364A/C genotype	—	—	—	—	—	—
AC/CC	1	—	1	—	1	—
AA	2.84 (1.45–5.55)	0.002	3.83 (1.40–10.5)	0.009	3.59 (1.47–8.80)*	0.005
Sex	—	—	—	—	—	—
Women	1	—	1	—	—	—
Men	0.75 (0.43–1.32)	0.318	0.92 (0.38–2.24)	0.860	—	—
Age, per 5 yr	0.99 (0.91–1.09)	0.898	0.96 (0.83–1.11)	0.578	—	—
SAPS II†	1.03 (1.01–1.05)	0.010	1.02 (0.99–1.05)	0.328	1.01 (0.98–1.03)	0.665
SOFA score‡	1.04 (0.99–1.08)	0.132	0.99 (0.88–1.10)	0.806	—	—
Body mass index, kg/m^2§	0.96 (0.90–1.02)	0.961	0.98 (0.90–1.06)	0.570	—	—
Continuous hemofiltration/ dialysis‖	—	—	—	—	—	—
No	1	—	1	—	1	—
Yes	3.32 (1.53–7.23)	0.003	2.22 (0.81–6.04)	0.120	2.76 (1.19–6.40)	0.018
Plasma angiotensin II, pg/ml#	1.81 (0.83–3.95)	0.830	0.99 (0.19–5.07)	0.990	—	—
Serum aldosterone, ng/l**	2.30 (1.38–3.85)	0.001	1.81 (0.74–4.43)	0.193	1.43 (0.76–2.66)	0.266
C-reactive protein, mg/dl††	0.84 (0.44–1.64)	0.618	0.70 (0.16–3.09)	0.640	—	—
IL-6, pg/ml#	2.06 (1.48–2.85)	<0.001	3.13 (1.42–6.88)	0.005	1.42 (0.92–2.18)	0.114
Procalcitonin, ng/ml#	1.39 (0.96–2.01)	0.085	0.84 (0.39–1.81)	0.664	—	—

The following variables were $\log_{10}$ transformed to address the skewness of distribution: plasma angiotensin II, serum aldosterone, C-reactive protein, interleukin 6 (IL-6), and procalcitonin. *P* values were determined using two-sided Wald tests.

* After the additional exclusion of SAPS II, serum aldosterone, and IL-6, the estimate changed to 4.46 (1.96–10.14), $P < 0.001$. † Four cases missing; ‡ Three cases missing; § 29 cases missing; ‖ 26 cases missing; # 19 cases missing; ** 20 cases missing; †† Five cases missing.

SAPS II = Simplified Acute Physiology Score II; SOFA = Sequential Organ Failure Assessment score.

−1364A/C polymorphism may have important effects on AQP5 expression in severe sepsis, it also suggests the potential relevance of AQP5 expression in severe sepsis.

Obviously, the underlying molecular and physiologic alterations linking decreased AQP5 expression to increased 30-day survival cannot be pinpointed by our study. These alterations remain to be elucidated at a basic research level. However, a few speculations can be made. In a previous study,[3] we described this novel, functional, and common single-nucleotide polymorphism in the AQP5 gene promoter as altering gene expression in different *in vitro* systems and cells. It was also found to alter RAAS regulation in young healthy men and individuals with coronary artery disease.[3] The C-allele was associated with decreased AQP5 expression and significant RAAS suppression in response to a high salt diet. In addition, RAAS suppression was significantly

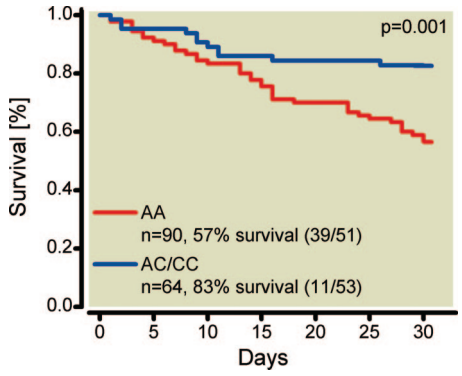

Fig. 1. Thirty-day survival in patients with severe sepsis. Kaplan–Meier estimates were used to calculate probabilities of 30-day survival based on aquaporin (AQP) 5 promoter −1364A/C polymorphism. Thirty-day survival was significantly decreased in AA genotypes compared with carriers of the C-allele.

Fig. 2. Thirty-day survival in patients with severe sepsis. Plasma angiotensin II concentration was not associated with 30-day survival.

blunted among individuals carrying the AA genotype. Because Hagiwara *et al.*[12,13] demonstrated significantly increased angiotensin II serum concentrations in lipopolysaccharide-induced systemic inflammation and because experimental studies[14,15] have considered angiotensin II to be a key mediator of inflammation—exerting proinflammatory effects by inducing the expression of cytokines, chemokines, adhesion molecules, growth factors, and increased reactive oxygen species concentrations—we speculated that the association of the C-allele with improved 30-day survival in severe sepsis may be the result of increased RAAS suppression in comparison with the AA genotype.

However, our data show for the first time that plasma angiotensin II and serum aldosterone concentrations in patients with severe sepsis are associated neither with the AQP5 −1364A/C polymorphism nor with 30-day survival. Of note, plasma angiotensin II concentration was two-fold higher in patients with severe sepsis in comparison with young healthy men, as described previously.[3] In contrast, serum aldosterone concentrations in patients with severe sepsis were not increased in comparison with those of young healthy men.[3]

A second and perhaps more provocative explanation of how decreased AQP5 gene expression may have enhanced survival in severe sepsis is that AQP5 expression also mediates cell migration, yet another inflammatory key mechanism that involves transient formation of membrane protrusions (lammellipodia and membrane ruffles) at the cell's leading edge.[2,16] Because AQP5 mediates the membrane protrusion *via* water entry, decreased AQP5 expression may reduce cell migration and proliferation and, therefore, alter the inflammatory chain during sepsis. Future mechanistic studies should, therefore, investigate whether the C-allele is also associated with attenuated migration and proliferation of inflammatory cells in response to stimuli relevant in severe sepsis.

The limitations of this investigation should be mentioned. Unrecognized selection bias, inherent to many genetic-association studies, cannot ultimately be excluded. Moreover, although all sepsis patients were treated with a rather standardized multimodal regimen, because of the multifactoral nature of this disorder, we cannot exclude the possibility that unknown and potentially confounding factors exist. Nevertheless, for the given indication, the study population was not small and multivariate Cox regression analyses revealed AQP5 −1364A/C polymorphism as an important and strong independent prognostic factor for 30-day survival in severe sepsis. This underscores the potential relevance of AQP5 expression in severe sepsis, regardless of the mechanisms involved.

Specific analyses of the behavior of plasma angiotensin II and serum aldosterone concentrations during the course of the disease are not provided. Plasma angiotensin II and serum aldosterone concentrations were measured once (*i.e.*, within 24 h after meeting severe sepsis criteria). Therefore, we can only state that the AQP5 −1364A/C polymorphism is not associated with plasma angiotensin II and serum aldosterone concentrations within the first 24 h after a patient meets severe sepsis criteria. Although repeated measurements of plasma angiotensin II and serum aldosterone concentrations during the course of sepsis may have expanded our findings, concentrations during disease course are likely to depend on myriad factors. Furthermore, because regional RAAS is at work in several organs, it is likely that operative hormone levels within the tissue are of greater import to organ and cellular function.

We report for the first time that AQP5 −1364A/C polymorphism is associated with increased survival in patients with severe sepsis, but not with plasma angiotensin II and serum aldosterone concentrations, within the first 24 h after patients meet severe sepsis criteria. Because the AQP5 −1364A/C polymorphism is associated with altered RAAS regulation in young healthy men and individuals with coronary artery disease, further sepsis studies are needed to investigate the influence of AQP5 −1364A/C polymorphism on RAAS during the disease course.

In conclusion, the C-allele of AQP5 −1364A/C polymorphism is associated with increased survival in patients with severe sepsis. Future studies are required to replicate these observations and to unravel the precise molecular mechanism by which the C-allele of the −1364A/C polymorphism influences survival in severe sepsis. It is our hope that this mechanism leads to the identification of novel treatment regimens that target AQPs or their expression.

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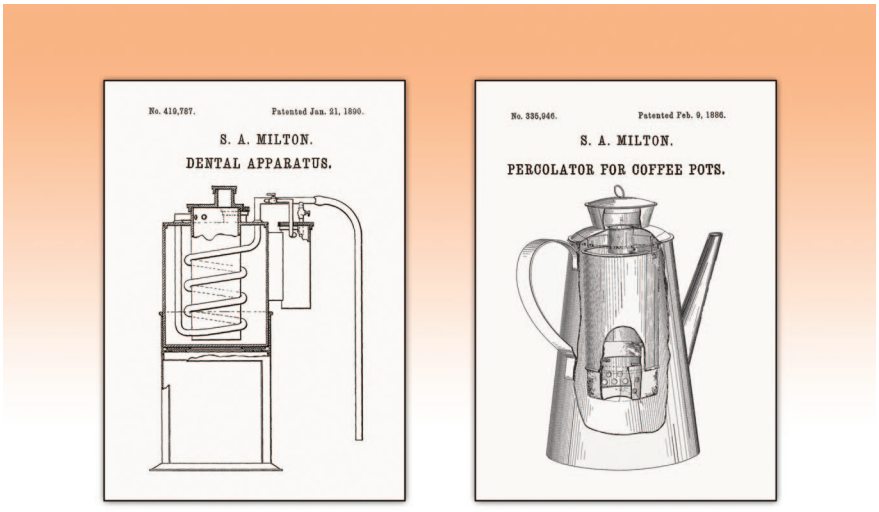
ANESTHESIOLOGY REFLECTIONS

The Milton Apparatus for Anesthetizing. . . and Awakening



A schoolteacher-turned-dentist, Samuel A. Milton (1847–1917) worked in his brother's dental practice from 1866 until completing a D.D.S. in 1880 at the Philadelphia Dental College. A decade later, he received a U.S. Patent for his apparatus (*left*) for charging "heated air or nitrous oxide . . . with the vapors of . . . [numbing] volatile oils" and conveying this medicated gas stream to the tooth of a patient. Note the smaller cylinder upstream for titrating in ether or chloroform to supplement the topical (oil of cloves) or inhaled (nitrous oxide) anesthetic effect of the gas stream applied to the oral cavity. Needless to say, the Milton Apparatus could expose dentist-anesthetists to more than trace anesthetic vapors. However, since Dr. Milton had patented his coffee percolator (*right*) nearly 4 yr before his anesthetic apparatus, he likely had some caffeinated assistance in shaking off any drowsiness from his apparatus' unscavenged anesthetic vapors. (Copyright © the American Society of Anesthesiologists, Inc. This image also appears in the *Anesthesiology Reflections* online collection available at www.anesthesiology.org.)

George S. Bause, M.D., M.P.H., Honorary Curator, ASA's Wood Library-Museum of Anesthesiology, Park Ridge, Illinois, and Clinical Associate Professor, Case Western Reserve University, Cleveland, Ohio. UJYC@aol.com.