19. Bradlow H, Telang N, Sepkovic D, et al. 2-hydroxyestrone: the 'good' estrogen. *J Endocrinol*. 1996;150:S259–S265.
20. Fishman J, Martucci C. Biological properties of 16alpha-hydroxyestrone: implications in estrogen physiology and pathophysiology. *J Clin Endocrinol Metab*. 1980;51:611–615.
21. Hankinson S, Willett W, Colditz GA, et al. Circulating concentrations of insulin-like growth factor I and risk of breast cancer. *Lancet*. 1998;351: 1393–1396.
22. Zhu B, Bui Q, Weisz J, et al. Conversion of estrone to 2- and 4-hydroxyestrone by hamster kidney and liver microsomes: implications for the mechanism of estrogen-induced carcinogenesis. *Endocrinology*. 1994;135:1772–1779.
23. Liehr J, Ricci M. 4-hydroxylation of estrogens as marker of human mammary tumors. *Proc Natl Acad Sci U S A*. 1996;93:3294–3296.
24. Ries LAG, Eisner MP, Kosary CL, et al. *SEER Cancer Statistics Review, 1973–1999.* Bethesda, MD: National Cancer Institute; 2002. http://seer.cancer.gov/csr/1973_1999/. Accessed July 8, 2003.

Genotyping Patients With Recent Blood Transfusions

Michelle Ng Gong,[*] Yang Sai,[†] Wei Zhou,[†] B. Taylor Thompson,[*] Li-Lian Xu,[†] and David C. Christiani[†]*

Background: Many studies have used polymerase chain reaction amplification (PCR) to genotype for common polymorphisms in intensive-care units (ICUs) where blood transfusions are common. Evidence that donor leukocytes in transfused blood can be detected by PCR of the recipient blood suggests that this minor population of donor white cells (microchimerism) can interfere with genotyping of allelic polymorphisms in critically ill transfused patients. To investigate this possibility, we assayed DNA extracted from the blood and buccal cells of ICU patients for 2 common polymorphisms in the *TNF-β* gene and the surfactant protein-B (*SP-B*) gene.

Methods: Study subjects were ICU patients from the Massachusetts General Hospital (Boston, MA) enrolled into a study on the molecular epidemiology of acute respiratory distress syndrome between January 1999 and October 2000. Blood and buccal cells were collected and DNA was extracted from 145 patients. Genotyping was performed by enzyme digestion and pyrosequencing.

Results: The Kappa statistics comparing the genotype results from blood and buccal cells were 0.98 (95% confidence interval [CI] = 0.94–1.01) for *TNFB* and 0.95 (CI = 0.87–1.02) for *SP-B*. When the analysis was restricted only to the 107 patients who were transfused, the Kappa statistic remained high at 0.97 (CI = 0.93–1.01) for *TNFB* and 0.93 (CI = 0.84–1.03) for *SP-B*.

Conclusion: We conclude that microchimerism from allogeneic blood transfusion is unlikely to have major effects on the genotype results of common polymorphisms in large molecular epidemiology studies conducted in the critical care setting if DNA is collected within a day after transfusions.

Key Words: blood transfusion, polymorphism (genetics), intensive-care unit, polymerase chain reaction

Submitted 23 July 2002; final version accepted 18 July 2003.

From the *Pulmonary and Critical Care Unit, Department of Medicine, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts; and the †Environmental Health Department, Harvard School of Public Health, Boston, Massachusetts.

This work was supported by research grants RO1 HL60710 and ES00002 from the National Institutes of Health. Dr. Gong is supported by K23 HL67197 from NHLBI and Drs. Xu and Miller by T32 ES07069 from NIEHS.

Correspondence: David C. Christiani, Harvard School of Public Health, 665 Huntington Avenue, Boston, MA 02115. E-mail: dchris@hohp.harvard.edu.

DOI: 10.1097/01.ede.0000091680.23304.21

Recently, genetic susceptibility to severe illnesses such as sepsis and acute respiratory distress syndrome (ARDS) has been investigated in intensive-care units (ICU).[1–6] All of these studies used polymerase chain reaction amplification (PCR) to amplify DNA extracted from peripherally drawn blood. PCR is highly sensitive. Experiments in which serially diluted DNA templates were amplified by PCR suggest that, theoretically, PCR has the ability to amplify even 1 molecule of DNA.[7]

Because of its sensitivity, PCR is vulnerable to contamination from extraneous DNA.[8] Transfusions might be one source of DNA contamination in critically ill patients. Donor leukocytes are a notable contaminant in packed red blood cells and are believed to be responsible for most transfusion reactions.[10] It is estimated that there are 10^9 leukocytes/unit of packed red blood cells or platelet.[11] Leukocyte-reduced blood components, which have been filtered to remove donor white cells, can have as much as 52 million leukocytes per unit of platelets and 250 million leukocytes per unit of packed red blood cells.[10] Plasma can have 30,000 copies of cell-free DNA per milliliter.[12] This has important implications for molecular epidemiology studies that use PCR in the critical care setting, because blood transfusions in the ICU are common, occurring in 65% to 85% of patients.[9,13]

The presence of a distinct minor white blood cell population (microchimerism) in recipients after allogeneic blood transfusion has been demonstrated by cytogenetics up to 1 week after transfusion.[14] Other studies using PCR have demonstrated persistence of donor DNA in recipients for 7 days[15–19] with a possible increase 3 or 4 days after transfusion from donor leukocyte proliferation.[16,17] One study found 0.5% to 10% of circulating leukocytes to be donor in origin.[19]

Given these findings, the possibility arises that in large molecular epidemiology studies of critically ill patients, genotype misclassification can occur because of PCR amplification of transfused DNA. To investigate this possibility, we compared the genotype results from the PCR amplification of DNA extracted from blood and buccal cells of 145 ICU patients enrolled in the Molecular Epidemiology of ARDS Study. Theoretically, buccal cells should not be contaminated with DNA from transfusions. We examined specifically 2 common polymorphisms: the *Nco*I restriction fragment length polymorphism in intron-1 of the *TNF-β gene* and a

variable nucleotide tandem repeat polymorphism in intron-4 of the *SP-B* gene.

METHODS

Study Population and Sample Collection

The subjects came from a cohort of adult ICU patients recruited between January 1999 and October 2000 from the Massachusetts General Hospital (Boston, MA) for a study of genetic susceptibility to ARDS. Any patients from the primary study with both blood and buccal cells available were included in this study. The Human Subjects Committees of the Massachusetts General Hospital and Harvard School of Public Health approved the study, and informed written consent was obtained from all subjects or their appropriate surrogates.

Buccal cells and one 10-mL edetic acid tube of blood were collected from each subject for DNA extraction. The patient's oral mucosa was cleaned with sterile saline and stroked with sterile cytology brushes that were then dipped into cell lysis solution from PureGene (Gentra Systems, Inc., Minneapolis, MN). Buccal cells were not obtained if there were signs of oral bleeding.

The transfusion history from the 8 days preceding the collection of blood samples was recorded from all subjects. Fresh-frozen plasma and cryoprecipitate were combined under plasma in this study.

DNA Extraction and Genotyping

DNA was extracted from peripheral blood mononuclear cells and buccal cells using PureGene kits according to the manufacturer's instructions. PCR amplification and the genotype procedure for *SP-B* were as previously described by Floros et al.[20] Genotyping was repeated in a random 10% of the samples as quality control. For the *TNF-β* gene, a 782-bp fragment of DNA was PCR-amplified as previously described.[3] Genotyping for *TNF-B* was performed by automatic DNA pyrosequencing using the SNP Reagent Kit 596 (Pyrosequencing Inc., Uppsala, Sweden) and the sequencing primer: 5′-CACACATTCTCTGTTTCTGCCA-3′. A guanine at position 329 indicates the *Nco*I+ TNFB1 allele, whereas adenine indicates *Nco*I-TNFB2.[21] Genotyping was repeated using *Nco*I enzyme digestion, as previously described, in a random 10% of the samples for quality assurance.[3] The laboratory was blinded to the transfusion history of the subjects. Three individuals performed the genotyping and each was blinded to results from the others. Genotyping for the *SP-B gene* was performed first. The genotyping for *TNF-β* was done later and included 34 additional patients who were enrolled into the study later.

Statistical Analysis

Agreement between genotype results of blood and buccal samples was calculated with a Kappa statistic. A forward stepwise logistic regression model predicting for discordant results between blood and buccal cells was used to determine the contribution of covariates such as the type, amount, and timing of transfusions, and genotype frequency.

RESULTS

Of the 145 patients in the study, 107 (74%) received transfusions (Table 1). Half the patients received multiple types of blood products, and 25% of the patients received 10 or more units of packed red blood cells or 18 or more units of platelets. Leukocyte-reduced blood and platelets were given to 14 (13%) of the transfused patients. More DNA was extracted from 10 mL of blood than from buccal cells, with a median yield of 31.5 μg/mL of blood versus 31.8 μg for all of the buccal cells. The allele frequency in the blood samples was 0.64 for *TNFB2* and 0.10 for the variant *SP-B*, and neither deviated substantially from Hardy Weinberg equilibrium. The genotype results for *TNF-β* and *SP-B* in the transfused patients are shown in Tables 2 and 3. All genotyping repeated for quality control did not differ from the initial genotyping.

There was 100% agreement between the blood and buccal cell genotype for both polymorphisms in the 38 patients who did not receive any transfusions. The Kappa statistics for the 145 patients in the study were 0.98 (95% confidence interval [CI] = 0.94–1.01) for *TNF-β* and 0.95 (CI = 0.87–1.02) for *SP-B*. When the analysis was restricted to the 107 patients who were transfused, the Kappa remained high at 0.97 (CI = 0.93–1.01) and 0.93 (CI = 0.84–1.03) for *TNF-β* and *SP-B*, respectively. In 1 transfused patient who received 2 units packed red blood cells 5 days before DNA

TABLE 1. Transfusion History of the 107 Transfused Patients in the 8 Days Before Sample Collection

	Packed Red Blood Cells	Fresh-Frozen Plasma	Platelets	Cryoprecipitate
Number of patients to receive product*; No. (%)	98 (92)	59 (55)	30 (28)	6 (6)
Units transfused; Median (range)	2 (1–50)	8 (1–70)	11 (6–50)	10 (1–14)
Days between sample collection and last transfusion; Median (range)	1 (0–8)	1 (0–7)	1 (0–8)	1 (0–7)

*Sum is greater than 107 because 53 patients (50%) received multiple types of blood products.

TABLE 2. Genotype Results for the *Nco*I Polymorphism of the *TNF-β* Gene

		Genotype of Buccal Cells*		
		TNFB1/ TNFB1	***TNFB1/ TNFB2***	***TNFB2/ TNFB2***
Genotype of Blood Sample	*TNFB1/TNFB1*	14	0	0
	TNFB1/TNFB2	0	48	1
	TNFB2/TNFB2	0	1	43

*Kappa = 0.97 (95% confidence interval = 0.93–1.01).

TABLE 3. Genotype Results for the Variant Polymorphism in Intron 4 of the SP-B Gene (No.)

		Genotype of Buccal Cells*		
		Wild/ Wild	**Insertion Variant**	**Deletion Variant**
Genotype of Blood Sample	Wild/wild	78	0	0
	Insertion variant	2	6	0
	Deletion variant	0	0	10

*Kappa = 0.93 (95% confidence interval = 0.84–1.03).

collection and 2 units of fresh-frozen plasma 2 days before DNA collection, the blood–buccal genotype results were discordant for both *TNF-β* and the *SP-B*. The 1 patient with discordant results for *TNF-β* received 2 units blood on the day of DNA collection, whereas the patient with discordant results for *SP-B* received 16 units blood, 8 units of fresh-frozen plasma, and 6 units of platelets on the day of DNA collection.

In the multivariable model, no variable was associated with disagreement between blood and buccal cell genotyping. However, the sample size was too small for the detection of any but very large effects.

DISCUSSION

We compared the DNA from blood and buccal cells to evaluate the possibility that microchimerism from transfusions might interfere with PCR-based genotyping of critically ill populations. The collection of buccal cells is time-consuming and the DNA yield is low. Thus, blood is a preferable source for DNA extraction and genotyping if it is reasonably certain that the genotype is not affected by transfusions.

Our study has several limitations. The patients were enrolled after blood transfusions had occurred; hence, donors were not available for genotyping. DNA was obtained within 1 day of the last blood transfusion. If donor leukocytes increase 3 to 4 days after transfusion as previously suggested,[17] it is possible that there might have been more discordant genotype results if DNA was collected later. Lastly, only 2 genes (*TNF-β* and *SP-B*) were genotyped because they were part of the primary investigation into the genetic susceptibility to ARDS. Genotyping other polymorphisms could add further confidence to our results.

Nevertheless, our study did demonstrate some interesting findings. We found no misleading genotype results from the blood in 98% of the patients transfused, even though massive transfusions were common in our patients. No factors related to the transfusion history or genotype frequencies were associated with discordance between blood and buccal genotypes in the multivariate model. However, with a limited sample size and only 3 outcomes, our study could not address this question definitively. Nevertheless, contamination from microchimerism appears to be a minor problem given the high level of agreement between the blood and buccal cell results.

It is possible that contamination from a source other than transfusions is responsible for the spurious result in our 3 discordant individuals. The patients were often mechanically ventilated and so their mouth care is limited. Some of these patients had been resuscitated in the field where mouth-to-mouth resuscitation could have been used. One patient was heterozygous for *TNFB1/2* by buccal cells and homozygous for *TNFB2* by blood, suggesting contamination of buccal cell collection. However, any contamination is unlikely to be systematic or common given the agreement between blood and buccal analysis, as well as conformity to Hardy-Weinberg equilibrium.

The difference between our results and previous reports of microchimerism can be explained by the different sensitivity of the PCR method used. In some studies, donor DNA was selectively amplified over the more plentiful recipient DNA by PCR primers that were specific for the donor but not recipient DNA.[15–19] In addition, the sensitivity of PCR was further increased in some studies by doing a nested PCR in which regions specific to the donor DNA are amplified twice with 2 pairs of primers.[16,18]

In this study, PCR was used to amplify genes that are common to recipients and donors. The recipient DNA would have been preferentially amplified over the donor DNA, because the recipient DNA constitutes most of the DNA pool. Using PCR techniques similar to those used in this study, Wenk et al. found identical pre- and posttransfusion genotype results for 9 polymorphisms in 10 patients after massive transfusions.[22] Likewise, Reid et al. correctly genotyped the blood group antigen for 60 patients transfused 2 to 50 units of packed red blood cells with no interference from microchimerism.[23]

In conclusion, there was very high agreement between the blood and the buccal cell genotype of critically ill patients

in this study. This suggests that for large-scale molecular epidemiology studies using PCR-based genotyping for allelic polymorphisms in patients who have been transfused with blood products, collection of blood for genotyping within a day of blood transfusion is sufficient.

ACKNOWLEDGMENTS

We thank Wei-Ling Zhang, Barbara Bean, and Linda Lineback for patient recruitment; Andrea Solomon for research support; Lucille Pothier for analytical assistance, Paige Williams for her statistical advice; and Li Su for laboratory support.

REFERENCES

1. Reid CL, Perrey C, Pravica V, et al. Genetic variation in proinflammatory and anti-inflammatory cytokine production in multiple organ dysfunction syndrome. *Crit Care Med*. 2002;30:2216–2221.
2. Mira JP, Cariou A, Grall F, et al. Association of *TNF2*, a TNF-α promoter polymorphism, with septic shock susceptibility and mortality. *JAMA*. 1999;282:561–568.
3. Stuber F, Petersen M, Bokelmann F, et al. A genomic polymorphism within the tumor necrosis factor locus influences plasma tumor necrosis factor-α concentrations and outcome of patients with severe sepsis. *Crit Care Med*. 1996;24:381–384.
4. Tang GJ, Huang SL, Yien HW, et al. Tumor necrosis factor gene polymorphism and septic shock in surgical infection. *Crit Care Med*. 2000;28:2733–2736.
5. Majetschak M, Flohe S, Obertacke U, et al. Relation of a TNF gene polymorphism to severe sepsis in trauma patients. *Ann Surg*. 1999;230: 207–214.
6. Marshall RP, Webb S, Bellingan GJ, et al. Angiotensin converting enzyme insertion/deletion polymorphism is associated with susceptibility and outcome in acute respiratory distress syndrome. *Am J Respir Crit Care Med*. 2002;166:646–650.
7. Saiki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with thermostable DNA polymerase. *Science*. 1988;239:487–491.
8. Reed W, Lee TH, Vichinsky EP, et al. Sample suitability for detection of minor white cell populations (microchimerism) by polymerase chain reaction. *Transfusion*. 1998;38:1041–1045.
9. Haller M, Forst H. Red cell transfusion therapy in the critical care setting. *Transfus Sci*. 1997;18:459–477.
10. Lane TA, Anderson KC, Goodnough LT, et al. Leukocyte reduction in blood component therapy. *Ann Intern Med*. 1992;117:151–162.
11. Pisciotti PT. *Blood Transfusion Therapy: A Physician's Handbook*, 3rd ed. Arlington: American Association of Blood Banks; 1989;8–15.
12. Lee TH, Montalvo L, Chrebtow V, et al. Quantitation of genomic DNA in plasma and serum samples: higher concentrations of genomic DNA found in serum than in plasma. *Transfusion*. 2001;41:276–282.
13. Vamvakas EC, Carven JH. RBC transfusion and postoperative length of stay in the hospital or the intensive care unit among patients undergoing coronary artery bypass surgery: the effects of confounding factors. *Transfusion*. 2000;40:832–839.
14. Schechter GP, Whang-Peng J, McFarland W. Circulation of donor lymphocytes after blood transfusion in man. *Blood*. 1977;49:651–656.
15. Kruskall MS, Lee TH, Assmann SF, et al. Survival of transfused donor white blood cells in HIV-infected recipients. *Blood*. 2001;98:272–279.
16. Adams PT, Davenport RD, Reardon DA, et al. Detection of circulating white blood cells in patients receiving multiple transfusions. *Blood*. 1992;80:551–555.
17. Lee TH, Donegan E, Slichter S, et al. Transient increase in circulating donor leukocytes after allogeneic transfusions in immunocompetent recipients compatible with donor cell proliferation. *Blood*. 1995;85: 1207–1214.
18. Carter AS, Bunce M, Cerundolo L, et al. Detection of microchimerism after allogeneic blood transfusion using nested polymerase chain reaction amplification with sequence-specific primers (PCR-SSP): A cautionary tale. *Blood*. 1998;92:683–689.
19. Lee TH, Paglieroni T, Ohto H, et al. Survival of donor leukocyte subpopulations in immunocompetent transfusion recipients: frequent long-term microchimerism in severe trauma patients. *Blood*. 1999;93: 3127–3139.
20. Floros J, Veletza SU, Kotikalapudi P, et al. Dinucleotide repeats in human surfactant protein-B gene and respiratory distress syndrome. *Biochem J*. 1995;305:583–590.
21. Messer G, Spengler U, Jung C, et al. Polymorphic structure of the tumor necrosis factor (TNF) locus: an NcoI polymorphism in the first intron of the human *TNF-β* gene correlates with a variant amino acid in position 26 and a reduced level of TNF-*B* production. *J Exp Med*. 1991;173: 209–219.
22. Wenk RE, Chiafari FA. DNA typing of recipient blood after massive transfusion. *Transfusion*. 1997;37:1108–1110.
23. Reid ME, Rios M, Powell VI, et al. DNA from blood samples can be used to genotype patients who have recently received a transfusion. *Transfusion*. 2000;40:48–53.