or respiratory complications except for hydrocephalus between the remifentanil and nonremifentanil groups.

Male sex, greater age, blood transfusion, and worse preoperative consciousness level were significantly associated with increased mortality, as in previous publications.[16–18] After adjustment for preoperative and intraoperative variables that are associated with outcome, use of remifentanil was an independent factor for low in-hospital mortality. Mortality was significantly lower in the remifentanil group even in patients with ruptured ICA with preoperative JCS grade 3 (11.8% vs. 22.7%; $P = 0.004$, Table 2), indicating a possible association between the use of remifentanil and lower mortality even in patients with preexisting brain damage and increased intracranial pressure because of rupture of aneurysms.

Limitations

Because our data were based on an administrative claims database, several limitations should be acknowledged and our results interpreted carefully. First, and most importantly, this is a nonrandomized retrospective study. Although we used propensity score matching to adjust for differences in baseline characteristics, the results could have been biased by several unmeasured confounders. For example, no data were available regarding the size and location of aneurysms, or clot thickness evaluated by computed tomography.[18] We did not have commonly used clinical grading scales for subarachnoid hemorrhage in the database such as Hunt & Hess grade, Fisher grade, or World Federation of Neurosurgical Societies grade estimated by GCS combined with the presence of motor deficit that were known to be closely associated with outcome after subarachnoid hemorrhage,[19] although we used JCS that was known to correlate well with GCS.[8] In the present study, worse JCS was significantly associated with higher mortality, confirming the legitimacy of using the JCS for propensity score matching. We also did not have records of surgical procedures that could have affected the patients' outcomes, such as temporary clipping, although we adjusted for the duration of anesthesia because of its presumed association with the surgical difficulty.

Second, we could not evaluate the doses of intraoperative anesthetics and concurrent effects of various other drugs that could potentially have affected the postoperative outcomes. Although our logistic regression analyses for other anesthetics showed that no other agents were significantly contributed to in-hospital mortality, further prospective studies are required to confirm the present results and explore the underlying mechanism behind the better postoperative outcome observed in the remifentanil group.

Third, we could not completely exclude the possibility that outcome was associated with individual anesthesia care providers' choices made according to their patients' preoperative physiological status. In the present study, we adjusted preoperative cardiovascular comorbidities between groups. However, we should be aware of other factors that could not be extracted from the present data set. For example, one may not choose remifentanil for those patients with serious physiological derangement due to lethally increased intracranial pressure. We excluded those who died within 3 days postoperatively before propensity score matching. Even if we included these patients in the analysis, results were not altered. Postoperative neurological, cardiovascular, pulmonary, and infectious complications did not differ significantly between groups except for hydrocephalus, which was more common in the remifentanil group. This observation indicates that preoperative and intraoperative physical status may not be significantly different between groups and cannot solely account for postoperative complications or mortality.

Fourth, our propensity score matching was based on complete pooling of the characteristics information across hospitals, which might have caused residual confounding by the site effect. We did not use the nonpooling approach (ie, matching patients in each hospital) because of the paucity of data available to support the propensity score estimation within each hospital alone.

Fifth, choice of drug may be somewhat correlated with quality of care, which may affect postoperative outcome. Generally, nitrous oxide is recognized as harmful to brain in neurosurgery, especially for those with increased intracranial pressure[20] (although recent studies had conflicting results[21,22]) and its use may represent inferior care that is associated with increased mortality. In our present data set, use of nitrous oxide was not associated with mortality. Similarly, those who did not choose to use remifentanil might have given poor intraoperative and postoperative care to the patients, which might have contributed to the poor outcomes. In the present study, hospital volume for ICA clipping had no significant effect on postoperative mortality. Besides, the remifentanil group did not show significant improvement in postoperative LOS and mortality among patients who underwent rectal cancer surgery extracted from the same database.[4] However, we should be aware of this possible bias, which cannot be excluded.

Speculations on the Neuroprotective Mechanisms of Remifentanil

There are a number of previous papers that describe the physiological/biochemical factors that could affect postoperative outcome for patients undergoing ICA clipping that may be modulated by the use of remifentanil. These include the suppression of stress-related endocrine responses, hyperglycemia, and inflammation.

Hyperglycemia in SAH is known to be associated with worse outcomes.[23,24] General anesthesia with remifentanil may suppress further increases in blood glucose during surgery[25,26] that could damage intact as well as ischemic neurons. Remifentanil with propofol suppresses intraoperative increases in blood glucose during craniotomy.[27]

Several lines of evidence indicate that remifentanil strongly suppresses surgical stress-related endocrine responses compared with fentanyl-based or sevoflurane

anesthesia.[25,26] This effect of remifentanil persists into the early postoperative period[25,28,29] and may serve better postoperative recovery.

In addition, several publications also suggest that remifentanil may suppress inflammatory reactions after surgical insults in rats,[30] mice,[31] and humans.[32] Systemic inflammation is known to be associated with worse postoperative outcomes[33] and suppression of postoperative inflammatory reactions in general anesthesia with remifentanil might be associated with better outcomes.

Furthermore, the use of remifentanil may provide better brain conditions for surgery. Opioids including fentanyl and remifentanil do not have any effects on carbon dioxide reactivity or intracranial pressure,[34,35] whereas volatile anesthetics contribute to brain swelling by their vasodilatory effect.[36] Remifentanil patients might be exposed to smaller amounts of volatile anesthetics than nonremifentanil patients and this may help to avoid brain swelling, which may be deleterious to the brain when intracranial pressure is increased due to SAH.[36,37]

In our previous report, remifentanil use was associated with shorter LOS in patients undergoing brain tumor resection without preoperative disorders in consciousness.[4] However, in the present study, use of remifentanil was not significantly associated with shorter LOS although preoperative and intraoperative factors associated with increased mortality were also associated with greater LOS. One possible reason for the present results is that there were more survivors among remifentanil patients than among nonremifentanil patients. Those patients were suspected to have greater brain damage and may have required prolonged intensive care. ICA clipping accompanied by decompression craniotomy required patients to stay in hospital until further surgery to repair their skull defect. These factors may have contributed to diminishing the difference in early postoperative recovery between the 2 groups. After surgery, patients may receive hypnotics/narcotics other than remifentanil for sedation and that can mask differences in recovery. Prolonged postoperative use of hypnotics is reported to be a cause of postoperative cognitive impairment.[38] Besides, generally longer postoperative LOS in Japan than in the United States due to differences in health care delivery systems[39] may also mask differences in patients' postoperative early recovery.

The above descriptions are speculations and based on the limitations described earlier, we should be prudent to make any definitive statement from the present study that may change our regular clinical practice before obtaining other prospective studies that confirm our present results.

CONCLUSIONS

In conclusion, this propensity score-matched retrospective survey in Japan indicates a possible association between remifentanil use and less postoperative mortality in patients undergoing clipping of ICAs. The present data should be confirmed by other prospective investigations before reaching any conclusions that could change our clinical practice.

REFERENCES

1. Kapila A, Glass PS, Jacobs JR, et al. Measured context-sensitive half-times of remifentanil and alfentanil. *Anesthesiology*. 1995;83:968–975.
2. Fodale V, Schifilliti D, Pratico C, et al. Remifentanil and the brain. *Acta Anaesthesiol Scand*. 2008;52:319–326.
3. Uchida K, Yasunaga H, Miyata H, et al. Impact of remifentanil introduction on practice patterns in general anesthesia. *J Anesth*. 2011;25:864–871.
4. Uchida K, Yasunaga H, Miyata H, et al. Impact of remifentanil use on early postoperative outcomes following brain tumor resection or rectal cancer surgery. *J Anesth*. 2012;26:711–720.
5. Warlow CP. Epidemiology of stroke. *Lancet*. 1998;352:S1–S4.
6. Inagawa T, Takahashi M, Aoki H, et al. Aneurysmal subarachnoid hemorrhage in Izumo City and Shimane Prefecture of Japan: outcome. *Stroke*. 1988;19:176–180.
7. Todd MM, Hindman BJ, Clarke WR, et al. Intraoperative Hypothermia for Aneurysm Surgery Trial (IHAST) Investigators: mild intraoperative hypothermia during surgery for intracranial aneurysm. *N Engl J Med*. 2005;352:135–145.
8. Ono K, Wada K, Takahara T, et al. Indications for computed tomography in patients with mild head injury. *Neurol Med Chir*. 2007;47:291–298.
9. Sumitani M, Uchida K, Yasunaga H, et al. Prevalence of malignant hyperthermia and relationship with anesthetics in Japan: data from the diagnosis procedure combination database. *Anesthesiology*. 2011;114:84–90.
10. Yasunaga H, Yanaihara H, Fuji K, et al. Impact of hospital volume on postoperative complications and in-hospital mortality following renal surgery: data from the Japanese diagnosis procedure combination database. *Urology*. 2010;76:548–552.
11. Yasunaga H, Horiguchi H, Kuwabara K, et al. Outcomes following laparoscopic or open distal gastrectomy for early-stage gastric cancer: a propensity-matched analysis. *Ann Surg*. 2013;257:640–646.
12. Claassen J, Vu A, Kreiter KT, et al. Effect of acute physiologic derangements on outcome after subarachnoid hemorrhage. *Crit Care Med*. 2004;32:832–838.
13. Connolly ES Jr, Rabinstein AA, Carhuapoma JR, et al. American Heart Association Stroke Council; Council on Cardiovascular Radiology and Intervention; Council on Cardiovascular Nursing; Council on Cardiovascular Surgery and Anesthesia; Council on Clinical Cardiology. Guidelines for the management of aneurysmal subarachnoid hemorrhage: a guideline for healthcare professionals from the American Heart Association/American Stroke Association. *Stroke*. 2012;43:1711–1737.
14. Rosenbaum PR, Rubin DB. Constructing a control group using multivariate matched sampling methods that incorporate the propensity score. *Am Stat*. 1985;39:33–38.
15. Hubbard AE, Ahern J, Fleischer NL, et al. To GEE or not to GEE: comparing population average and mixed models for estimating the associations between neighborhood risk factors and health. *Epidemiology*. 2010;21:467–474.
16. Britz GW, Salem L, Newell DW, et al. Impact of surgical clipping on survival in unruptured and ruptured cerebral aneurysms: a population-based study. *Stroke*. 2004;35:1399–1403.
17. Naidech AM, Jovanovic B, Wartenberg KE, et al. Higher hemoglobin is associated with improved outcome after subarachnoid hemorrhage. *Crit Care Med*. 2007;35:2383–2389.
18. Rosengart AJ, Schultheiss KE, Tolentino J, et al. Prognostic factors for outcome in patients with aneurysmal subarachnoid hemorrhage. *Stroke*. 2007;38:2315–2321.
19. Ogilvy CS, Carter BS. A proposed comprehensive grading system to predict outcome for surgical management of intracranial aneurysms. *Neurosurgery*. 1998;42:959–968.

20. Reinstrup P, Messeter K. Cerebrovascular response to nitrous oxide. *Acta Anaesthesiol Scand.* 1994;38:761–762.
21. McGregor DG, Lanier WL, Pasternak JJ, et al. Intraoperative Hypothermia for Aneurysm Surgery Trial Investigators. Effect of nitrous oxide on neurologic and neuropsychological function after intracranial aneurysm surgery. *Anesthesiology.* 2008;108:568–579.
22. Pasternak JJ, McGregor DG, Lanier WL, et al. IHAST Investigators. Effect of nitrous oxide use on long-term neurologic and neuropsychological outcome in patients who received temporary proximal artery occlusion during cerebral aneurysm clipping surgery. *Anesthesiology.* 2009;110:563–573.
23. Kruyt ND, Biessels GJ, de Haan RJ, et al. Hyperglycemia and clinical outcome in aneurysmal subarachnoid hemorrhage: a meta-analysis. *Stroke.* 2009;40:e424–e430.
24. Latorre JG, Chou SH, Nogueira RG, et al. Effective glycemic control with aggressive hyperglycemia management is associated with improved outcome in aneurysmal subarachnoid hemorrhage. *Stroke.* 2009;40:1644–1652.
25. Ihn CH, Joo JD, Choi JW, et al. Comparison of stress hormone response, interleukin-6 and anaesthetic characteristics of two anaesthetic techniques: volatile induction and maintenance of anaesthesia using sevoflurane versus total intravenous anaesthesia using propofol and remifentanil. *J Int Med Res.* 2009;37:1760–1771.
26. Weale NK, Rogers CA, Cooper R, et al. Effect of remifentanil infusion rate on stress response to the pre-bypass phase of pediatric cardiac surgery. *Br J Anaesth.* 2004;92:187–194.
27. Cok OY, Ozkose Z, Pasaoglu H, et al. Glucose response during craniotomy: propofol-remifentanil versus isoflurane-remifentanil. *Minerva Anestesiol.* 2011;77:1141–1148.
28. Kabon B, Kugener A, Gruenberger T, et al. Effects of continuous remifentanil administration on intra-operative subcutaneous tissue oxygen tension. *Anaesthesia.* 2007;62:1101–1109.
29. Winterhalter M, Brandl K, Rahe-Meyer N, et al. Endocrine stress response and inflammatory activation during CABG surgery. A randomized trial comparing remifentanil infusion to intermittent fentanyl. *Eur J Anaesthesiol.* 2008;25:326–335.
30. Hasegawa A, Iwasaka H, Hagiwara S, et al. Remifentanil and glucose suppress inflammation in a rat model of surgical stress. *Surg Today.* 2011;41:1617–1621.
31. Zongze Z, Jia Z, Chang C, et al. Protective effects of remifentanil on septic mice. *Mol Biol Rep.* 2010;37:2803–2808.
32. Ke JJ, Zhan J, Feng XB, et al. A comparison of the effect of total intravenous anaesthesia with propofol and remifentanil and inhalational anaesthesia with isoflurane on the release of pro- and anti-inflammatory cytokines in patients undergoing open cholecystectomy. *Anaesth Intensive Care.* 2008;36:74–78.
33. Neal CP, Mann CD, Garcea G, et al. Preoperative systemic inflammation and infectious complications after resection of colorectal liver metastases. *Arch Surg.* 2011;146:471–478.
34. Petersen KD, Landsfeldt U, Cold GE, et al. Intracranial pressure and cerebral hemodynamic in patients with cerebral tumors: a randomized prospective study of patients subjected to craniotomy in propofol-fentanyl, isoflurane-fentanyl or sevoflurane-fentanyl anesthesia. *Anesthesiology.* 2003;98:329–336.
35. Ostapkovich ND, Baker KZ, Fogarty-Mack P, et al. Cerebral blood flow and CO_2 reactivity is similar during remifentanil/N_2O and fentanyl/N_2O anesthesia. *Anesthesiology.* 1998;89:358–363.
36. Matta BF, Heath KJ, Tipping K, et al. Direct cerebral vasodilatory effects of sevoflurane and isoflurane. *Anesthesiology.* 1999;91:677–680.
37. Sakabe T, Nakakimura K. Effects of anesthetic agents and other drugs on cerebral blood flow, metabolism and intracranial pressure. In: Cottrell JE, Smith DS, eds. *Anesthesia and Neurosurgery.* St. Louis: Mosby; 1994:149–174.
38. Pandharipande P, Shintani A, Peterson J, et al. Lorazepam is an independent risk factor for transitioning to delirium in intensive care unit patients. *Anesthesiology.* 2006;104:21–26.
39. Yim AP, Arifi AA, Wan S. Coronary artery bypass grafting in the elderly: the challenge and the opportunity. *Chest.* 2000;117:1220–1221.

Section Editor: Avery Tung

The Effects on Gastric Emptying and Carbohydrate Loading of an Oral Nutritional Supplement and an Oral Rehydration Solution: A Crossover Study with Magnetic Resonance Imaging

Makoto Nakamura, MD,* Kanji Uchida, MD, PhD,* Masaaki Akahane, MD, PhD,† Yasushi Watanabe,† Kuni Ohtomo, MD, PhD,† and Yoshitsugu Yamada, MD, PhD*

BACKGROUND: Preoperative administration of clear fluids by mouth has recently been endorsed as a way to improve postoperative outcomes. A carbohydrate-containing beverage supplemented with electrolytes or proteins may have additional benefits for patients' satisfaction. However, effects on gastric residual, nausea, and emesis and the effectiveness of these beverages for improving patients' hydration status have not been well defined.

METHODS: We evaluated changes in gastric volume over time by magnetic resonance imaging, as well as blood glucose levels, before and after administration of 500 mL oral rehydration solution (ORS) containing 1.8% glucose and electrolytes in 10 healthy volunteers. The same volume of an oral nutritional supplement (ONS) containing 18% glucose and supplemental arginine (545 mOsm/kg) was given to the same population using a crossover design.

RESULTS: The mean (median, 95% confidence interval) gastric fluid volume at 1 hour after oral ingestion was 55.0 (55.3, 39.0–70.9) mL in the ORS group, whereas 409.2 (410.9, 371.4–447.0) mL in the ONS group ($P = 0.0002$). The gastric fluid volume of all participants in the ORS group returned to <1 mL/kg at 90 minutes after ingestion, whereas none reached <1 mL/kg at 120 minutes in the ONS group. The ONS group showed a sustained increase in the blood glucose level after ingestion ($P < 0.0001$ to baseline at 30, 60, 120 minutes), while the ORS group showed an initial increase ($P < 0.0001$, $P = 0.01$, $P = 0.205$ at each time point).

CONCLUSIONS: ORS supplemented with a small amount of glucose showed faster gastric emptying, which may make it suitable for preoperative administration. In contrast, ONS supplemented with arginine with a relatively low osmolality was associated with a longer time for gastric emptying, although it showed a sustained increase in blood glucose level. (Anesth Analg 2014;118:1268–73)

During the past decade, based on the assumption that so-called clear fluids should be cleared from the stomach by 2 to 3 hours, several international guidelines[1–4] shortened preoperative fasting periods for clear fluids from overnight (>10 hours) to 2 to 3 hours before induction of anesthesia, though neither a strict formula for clear fluids nor a maximum safe volume for preoperative administration has been determined.

Recently, preoperative administration of fluid supplemented with glucose has even been endorsed to restore dehydration, ameliorate patients' discomfort, and reduce the incidence of postoperative insulin resistance,[5–9] which should improve overall postoperative outcomes. After the announcement of the Enhanced Recovery After Surgery protocol, an additional nutritional supplement in the beverages has been preferred for preoperative use; 400 mL of a 12.5% carbohydrate-containing drink has been shown to be safe in patients undergoing major abdominal surgery.[5,10–14]

Glucose-supplemented oral rehydration solution (ORS) (OS-1®, Otsuka Pharmaceutical Factory, Tokushima, Japan) contains both salts and glucose, and its osmolality is similar to that of serum; it is advocated for hydration purposes, especially for dehydrated patients. The beverage is assumed to be quickly eliminated from the stomach and is preferable for preoperative hydration for patients undergoing surgery. ORS is reported to be safe for preoperative use in the low-risk Japanese surgical population,[15] although the precise performance of ingested beverages in vivo has not been reported.

An arginine-containing oral nutritional supplement (ONS) (Arginaid Water®, Nestle Health Science Company, Tokyo, Japan) is a clear fluid supplemented with 18% glucose, 2% protein including arginine, and 0.008% zinc, and it

From the Departments of *Anesthesiology and Pain Relief Center and †Radiology, The University of Tokyo Hospital, Tokyo, Japan.

Accepted for publication August 13, 2013.

Funding: a Grant-in-Aid for Scientific Research A from the Ministry of Education and Science (No. 23249072, YY, KU), and a Grant-in-Aid for Scientific Research B from the Ministry of Education and Science (No. 24390364,KU). A Grant for "Rare Lung Diseases" from the Ministry of Health Labour and Welfare (H24-Nanchitou [Nanchi]-Ippan-035, KU).

Conflict of Interest: See Disclosures at the end of the article.

This report was previously presented, in part, at the 32nd Annual Meeting of the Japan Society of Clinical Anesthesia.

Reprints will not be available from the authors.

Address correspondence to Kanji Uchida, MD, PhD, Department of Anesthesiology and Pain Relief Center, The University of Tokyo Hospital. 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. Address e-mail to uchidakane@h.u-tokyo.ac.jp.

DOI: 10.1213/ANE.0b013e3182a9956f

has an osmolality of 545 mOsm/kg. Its calorie (0.8 kcal/mL) and glucose content (18%) are sufficient to fulfill the Enhanced Recovery After Surgery protocol.[16] Arginine facilitates healing from pressure ulcers[17] and from surgical wounds,[18] and it enhances the immunity of trauma patients.[19] Serum zinc decreases after surgery,[20] and zinc supplementation has been reported to be protective in an endotoxemic mouse model.[21] Therefore, perioperative administration of this beverage may help patients recover faster after surgery. However, the relatively higher glucose content and additives such as arginine in the beverage might compromise gastric emptying. There has been a limited number of papers on the efficacy and safety of this beverage in terms of gastric emptying.[22]

Magnetic resonance imaging (MRI) creates high-resolution images that distinguish gastric liquid from air and gastric wall, allowing direct measurement of gastric volume to be determined in real time.[10,23–25]

In the present study, the time course changes in gastric fluid volume (GFV) were evaluated by MRI, and carbohydrate loading efficiency was evaluated by measuring blood glucose levels after the administration of either ORS or ONS to 10 healthy volunteers in a crossover design to validate the efficacy and safety of these 2 beverages for preoperative use.

METHODS

Study Design, Setting, and Ethics

The protocol for this randomized, double-blind, crossover study involving healthy volunteers was approved by the Ethics Committee of the Graduate School of Medicine, University of Tokyo (IRB#3476), and it was conducted in accordance with the Declaration of Helsinki-Ethical Principles for Medical Research Involving Human Subjects and with the Ethical Guidelines for Clinical Research issued by the Ministry of Health, Labour, and Welfare in Japan. Written, informed consent was obtained from all participants before enrollment.

Participants

Ten healthy adult men (28–53 years), with no risk factors for delayed gastric emptying (e.g., morbidly obese, diabetes mellitus on medical treatment, past gastrointestinal disorders that required in-hospital treatment) and suitable for MRI scanning (e.g., no metal/implants in the body), were studied. A medical questionnaire was administered before recruitment to confirm the participants' background.

Interventions

Each participant was randomized in a crossover manner to groups ONS (Arginaid Water® Nestle Healthcare Science Company, Tokyo, Japan) or ORS (OS-1®, Otsuka Pharmaceutical Factory, Tokushima, Japan) (Fig. 1). The composition of each test solution is shown in Table 1. Participants were asked to drink 500 mL either OS-1® (ORS group) or Arginaid Water® (ONS group) on 2 separate occasions >5 days apart. Participants were instructed to abstain from alcohol, caffeine, medications, and strenuous exercise after 9:00 PM of the day before examination. They were asked to avoid food after 8:00 AM (>10 hours before ingestion) and beverages after 12 noon on the day of examination (>6 hours before ingestion). The time to ingest test drinks was set at approximately 6:00 PM. Before ingestion, participants' height, weight, and blood glucose levels were measured, followed by MRI scanning. Participants then consumed either beverage poured in paper cups in a sitting position within 3 minutes. MRI scans were done at 30, 60, 90, and 120 minutes after ingestion. Blood glucose levels were measured with the finger-prick method using Medisafe-mini® (Terumo Corporation, Tokyo, Japan) at 30, 60, and 120 minutes after ingestion.

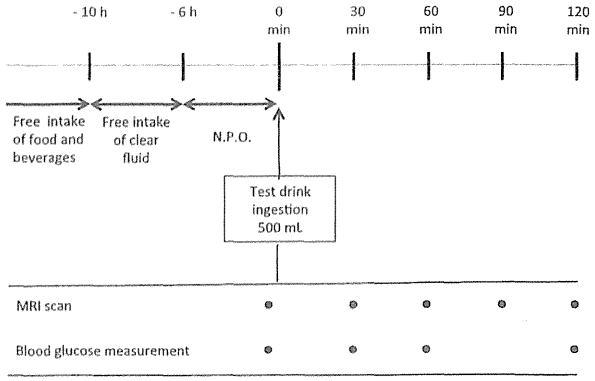


Figure 1. Schematic illustration of the experimental procedures. Closed circles indicate measurement or sampling. All participants in the present study started ingestion of beverages (0 minutes in the schematic) at approximately 6:00 PM.

Table 1. Composition of Each Test Fluid

	ONS-oral nutritional supplement, (Nestle Health Science Company, Arginaid Water®)	ORS-oral rehydration solution, (Otsuka Pharmaceutical Factory, OS-1®)
Energy (kcal/mL)	0.8	0.1
Carbohydrate (%)	18	2.5
Glucose (%)		1.8
Sodium (mEq/L)	0	50
Potassium (mEq/L)	—	20
Magnesium (mEq/L)	—	2
Lactate (mEq/L)	—	31
Chloride (mEq/L)	—	50
Phosphorus (mEq/L)	0.18	2
Zinc (%)	0.008	—
Copper (%)	0.0008	—
L-Arginine (%)	2	—
Osmolality (mOsm/L)	545	270

MRI Studies

All MRI scans were performed on 1.5-Tesla unit (MAGNETOM Avanto, Siemens Medical Solutions, Erlangen, Germany) at the University of Tokyo Hospital by a single professional operator who did not know which beverage was ingested. Each participant was positioned supine in the unit, and a coarse scout scan was taken to locate the position of the abdominal organs. Then, the half-Fourier acquisition single-short turbo spin echo (HASTE) sequence was used to acquire T2-weighed transverse images of the stomach with the following conditions: TE 83 milliseconds, field of view 350 mm, slice width 5 mm, gap width 1.5 mm, image matrix 205 × 256. Twenty-eight slices

were taken at each time point under a single breath-hold for 19.6 seconds.

All images were transferred to a personal computer in DICOM format and viewed to locate the gastric lumen and GFV. An example of a series of images throughout the abdomen from 1 participant is shown in Figure 2. The liquid content in the stomach in each slice was manually outlined as the area of interest using Osirix imaging software (Pixmeo SARL, Bernex, Switzerland) (Version 4.0, downloaded from: http://www.osirix-viewer.com) by 1 investigator blinded to the ingested beverages. The volume of gastric content was obtained by summing the volumes calculated from each slice; the area of interest in each slice was multiplied by the thickness of each slice.

End Points and Sample Size

The primary end point of the study was the remaining GFV at 60 minutes after oral ingestion measured on MRI. Secondary end points included blood glucose at each time point.

Based on the previous report by Lobo et al.,[10] the mean difference in GFV 60 minutes after oral ingestion was anticipated to be 80 mL, with a standard deviation (SD) of 50 mL, between groups ONS and ORS. Assuming an α error of 0.05 and a power of 90%, the sample size was calculated to be 10 per beverage.

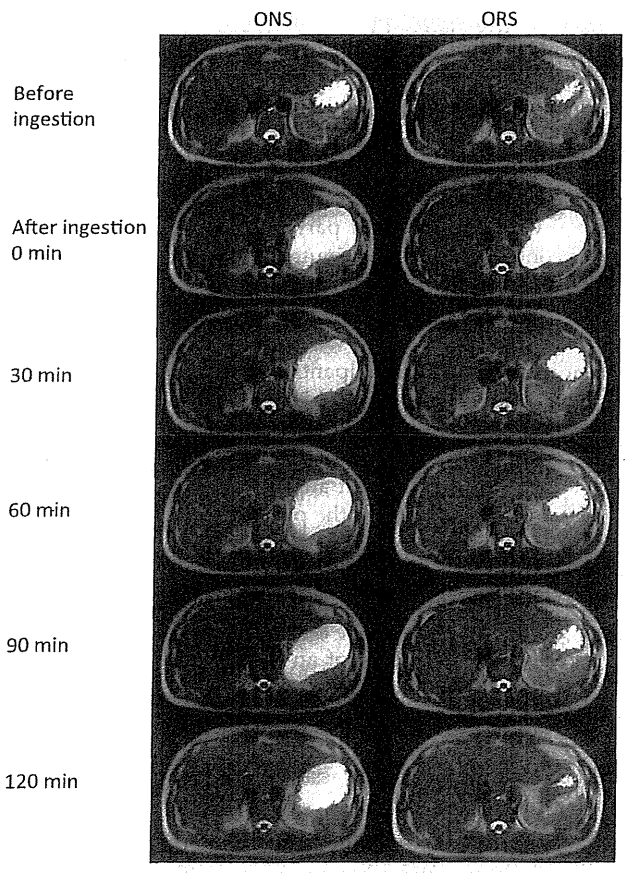


Figure 2. Sequential axial magnetic resonance imaging scans in 1 participant before and after ingestion of the drink. ONS = oral nutritional supplement (Arginaid Water ®); ORS = oral rehydration solution (OS-1®).

Statistical Analysis

All results are expressed as medians (interquartile range; IQR) except where noted. The Wilcoxon rank-sum test was used to compare differences of GFV at 60 minutes after ingestion between groups and blood glucose at each time point. Two-tailed *P*-value of 0.05 indicated significance for GFV at 60 minutes. Corrected *P*-value of 0.0125 was applied to indicate statistical significance for blood glucose at each time point to avoid a type I error for multiple comparisons between groups. The time course differences in blood glucose levels were compared using Dunn test with the pre-ingestion value as the control. Statistical analysis was done with JMP Pro v 9 for Macintosh (SAS Institute Inc., Cary, NC).

RESULTS

All volunteers were men. The median (IQR) age, weight, height, and body mass index of the 10 volunteers were 31.5 (27.8–34.3) years, 64.5 (61–70) kg, 169.5 (166.8–174.5) cm, and 21.9 (21.1–24.9) kg/m^2, respectively. No participant had underlying medical problems known to delay gastric emptying. All participants completed serial studies with 2 beverages, and no side effects were observed.

The fasting period and residual gastric volume before entering the study did not differ significantly between the groups: 6.5 (6.50–6.54) hours vs 6.5 (6.4–7.0) hours (P = 0.587), and 15 (8.3–38.2) mL vs 10.4 (6.1–23.3) mL (P = 0.4055) in the ORS and ONS group, respectively.

The GFV

After ingestion of the test drinks, the ORS group showed an immediate decrease in GFV, with less intersubject variation (Fig. 3A). GFV of all participants reached <1 mL/kg at 90 minutes (Fig. 4). In contrast, the ONS group showed varied patterns in decreasing GFV among participants (Fig. 3B). GFV did not reach <1 mL/kg at 120 minutes after ingestion in any participant (Fig. 4). The remaining GFV at 60 minutes was significantly higher (P = 0.0002) in the ONS group

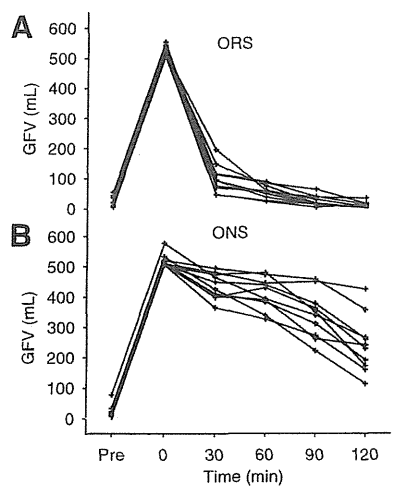


Figure 3. Individual residual gastric fluid volumes before and after ingestion (0, 30, 60, 90, and 120 minutes). A: ORS = oral rehydration solution. B: ONS = oral nutritional supplement. Individual gastric fluid volume kinetics is shown with symbols connected with solid lines. Volume at 0 minutes is the sum of the ingested volume (500 mL) and gastric fluid volume before ingestion. The gray line indicates gastric fluid volume as 0 mL. GFV = gastric fluid volume.

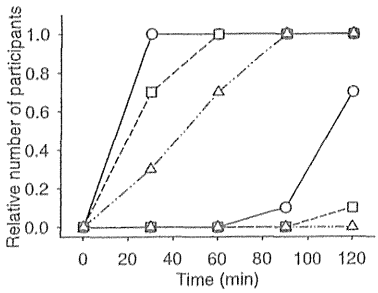


Figure 4. Cumulative fraction of participants ($n/10$) with body weight-corrected gastric fluid volume (GFVw) at each time point after oral fluid ingestion. Open symbols = oral nutritional supplement (Artinaid Water®); Gray symbols = oral rehydration solution (OS-1®); Circles = GFVw <50% of corresponding maximum GFVw after ingestion; Squares = GFVw <2 mL/kg; Triangles = GFVw <1 mL/kg. The gray dotted line indicates fraction 1.0.

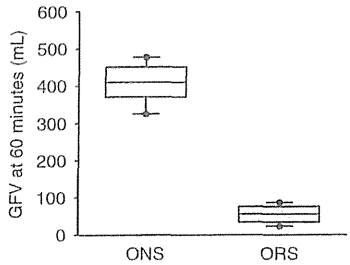


Figure 5. Residual gastric fluid volume (GFV) at 60 minutes after oral ingestion. Box plot of gastric residual volume for either oral nutritional supplement (ONS; Arginaid Water®) or oral rehydration solution (ORS; OS-1®) at 60 minutes after oral ingestion. Each vertical box with error bars represents 10th, 25th median and 75th and 90th percentile of GFV in each group. Closed dots indicate maximum and minimum GFV.

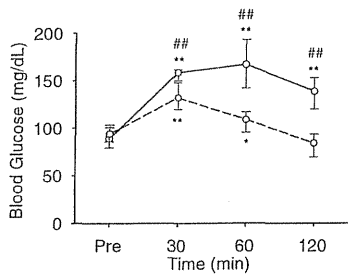


Figure 6. Blood glucose levels in the 2 groups. Open circles = oral nutritional supplement; Gray circles = oral rehydration solution. $*P = 0.01$ to baseline, $**P < 0.0001$ to baseline (i.e. before ingestion), $\#\#P < 0.001$ between 2 groups at the same timepoint ($P = 0.0009$, 0.0003, and 0.0002, at 30, 60, and 120 minutes, respectively).

(410.9, 371.2–451.6 mL, 95% confidence intervals (CIs) of means (95% CI, 371.4–447.0 mL) than in the ORS group (55.3, 34.1–76.6 mL, 95% CI, 39.0–70.9 mL) (Fig. 5).

Blood Glucose Levels

Baseline blood glucose levels before drink ingestion were 94 (85.8–103.5) mg/dL in the ORS group and 89 (79.3–100.8) in the ONS group, with no significant difference between groups ($P = 0.7618$). At 30 minutes after ingestion, blood glucose levels increased significantly in both groups compared with baseline ($P < 0.0001$). Blood glucose levels in the ORS group subsequently decreased and returned to baseline by 120 minutes ($P = 0.205$), whereas they remained significantly higher during the observation period in the ONS group ($P < 0.0001$ at 30, 60, 120 minutes). The ONS group showed significantly higher blood glucose levels than the ORS group throughout the observation period ($P = 0.0009$, 0.0003, 0.0002, at 30, 60, 120 minutes, respectively) (Fig. 6).

DISCUSSION

In the present study, precise MRI-based evaluation of remaining gastric fluid contents after oral administration of ORS showed quicker emptying with small variation among participants, while the ONS group showed delayed and variable emptying. Blood glucose levels were significantly increased in both groups, with sustained elevation in the ONS group.

The rate of transfer of fluid-containing nutrients from the stomach to the duodenum is reported to be 1.5–3 kcal/min,[26] and the present results of 1.9 kcal/min for the ONS group were within that range. Vist and Maughan[27] reported a mean ± SE half gastric emptying time of 600 mL (approximately 8 mL/kg) of a beverage with 18.8% glucose as 64 ± 8 minutes with lower osmolality (237 mOsm/kg) and as 130 ± 18 minutes with higher osmolality (1300 mOsm/kg) for healthy men volunteers. In the present study, 6 of 10 participants cleared half of the ingested 500 mL ONS with 18% glucose and an osmolality 545 mOsm/kg between 90 and 120 minutes (Fig. 4), which indicates that the clearance of ONS used in the present study was close to the high osmolar beverage in Vist and Maughan's report. Proteins in the ONS should stimulate intraluminal gastrin release[28] and increase gastric secretion significantly,[29] which may further increase GFV. Seven of 10 participants cleared >250 mL gastric content within 120 minutes, and that coincides with the carbohydrate-containing drink with the same calorie content (200 kcal).[10] Therefore, we anticipated that having patients drink 250 mL ONS would be safe for preoperative use. However, 2 of 10 participants could clear up to only 160 mL ONS, and clinicians should therefore be aware of the individual variation in gastric emptying when giving ONS. There are other beverages categorized as clear fluids that have a carbohydrate content and osmolality that are similar to those of ONS used in the present study.[30] It is recommended that the gastric emptying time of these beverages be individually evaluated to provide safe preoperative use.

In contrast to the ONS group, the ORS group showed faster gastric clearance. Vist and Maughan[27] reported the time required to clear half of an isotonic (230 mOsm/kg) glucose solution (4%) as 17 ± 1 minutes, and ORS in the present study showed similar results. Minimal individual variation between groups supports the notion that ORS might be suitable for quick passage from the stomach to the duodenum and ileum.

In Nygren's[5] report, blood glucose increased to 162 ± 7.2 mg/dL 40 minutes after ingestion of 400 mL carbohydrate-rich drink (285 mOsm/kg, 12.0% carbohydrate = 48 g, 0.46 mg/mL sodium). The 48 g carbohydrates led to an increase in insulin sufficient to fully depress hepatic glucose

production.[31] In the present study, 8 of 10 volunteers in the ONS group had blood glucose levels similar to those of the carbohydrate-rich drink group in Nygren's report. Therefore, ONS ingestion may be effective for reversing participants' metabolic status to the fed state.

Limitations of the Present Study

In the present study, the participants were healthy, relatively young (33 ± 7.6 years), Japanese men. Although the present results duplicate those of previous reports from Western countries, applying the present results to women,[10,32,33] children, older patients, and other ethnic groups should be done with caution. Special care should also be taken with patients with other comorbidities related to delayed gastric motility.

It is important to note that one cannot simply extrapolate the present data to patients before surgery, since patients have a certain degree of anxiety that may disturb rapid fluid clearance from the stomach, although that may not affect gastric emptying time.[5]

Effects of circadian rhythm should also be considered since the present study was done in the evening, whereas most preoperative patients undergo anesthesia in the morning. Although gastric fluid clearance rate is reported to be unchanged between morning and evening,[34] the insulin response to orally administered glucose is generally slower and delayed in the evening.[35]

Conclusions

In conclusion, glucose-supplemented ORS showed faster gastric emptying, which may make it suitable for preoperative administration without increasing the risk of GFV. In contrast, ONS supplemented with arginine with a relatively low osmolality was associated with a longer time for gastric emptying although it showed a sustained increase in blood glucose level that may reverse patients' metabolic status to the fed state. The use of preoperative oral fluids supplemented with glucose and protein should be tailored to each patient's specific needs and based on the safety profile in that patient population.

DISCLOSURES

Name: Makoto Nakamura, MD.
Contribution: This author helped design and conduct the study, analyze the data, and write the manuscript.
Attestation: Makoto Nakamura has seen the original study data, reviewed the analysis of the data, approved the final manuscript, and is the author responsible for archiving the study files.
Conflicts of Interest: The author has no conflicts of interest to declare.
Name: Kanji Uchida, MD, PhD.
Contribution: This author helped design and conduct the study, analyze the data, and write the manuscript.
Attestation: Kanji Uchida has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.
Conflicts of Interest: The author has no conflicts of interest to declare.
Name: Masaaki Akahane, MD, PhD.
Contribution: This author helped design and conduct the study and write the manuscript.
Attestation: Masaaki Akahane has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.
Conflicts of Interest: The author has no conflicts of interest to declare.
Name: Yasushi Watanabe.
Contribution: This author helped design and conduct the study.
Attestation: Yasushi Watanabe has seen the original study data and approved the final manuscript.
Conflicts of Interest: The author has no conflicts of interest to declare.
Name: Kuni Ohtomo, MD, PhD.
Contribution: This author helped design the study and write the manuscript.
Attestation: Kuni Ohtomo has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.
Conflicts of Interest: Kuni Ohtomo received research funding from Otsuka Pharmaceutical Factory (Tokushima, Japan).
Name: Yoshitsugu Yamada, MD, PhD.
Contribution: This author helped design and conduct the study, analyze the data, and write the manuscript.
Attestation: Yoshitsugu Yamada has seen the original study data, reviewed the analysis of the data, and approved the final manuscript.
Conflicts of Interest: Yoshitsugu Yamada received research funding from Otsuka Pharmaceutical Factory (Tokushima, Japan) and received research funding from Nestle Health Science Company (Tokyo, Japan).
This manuscript was handled by: Steven L. Shafer, MD.

ACKNOWLEDGMENTS

The authors would like to thank Kenichi Shukuya and Yutaka Yatomi, MD, PhD, for their technical support and sharing their knowledge, and Drs. M. Otsuji, MD, PhD, M. Bougaki, MD, PhD, M. Muroya, MD, PhD, and M. Maekawa, MD, for their help in preparation of the project.

REFERENCES

1. Practice guidelines for preoperative fasting and the use of pharmacological agents for the prevention of pulmonary aspiration: application to healthy patients undergoing elective procedures: a report by the American Society of Anesthesiologist Task Force on Preoperative Fasting. Anesthesiology 1999;90:896–905
2. Ljungqvist O, Søreide E. Preoperative fasting. Br J Surg 2003;90:400–6
3. Søreide E, Eriksson LI, Hirlekar G, Eriksson H, Henneberg SW, Sandin R, Raeder J; (Task Force on Scandinavian Pre-operative Fasting Guidelines, Clinical Practice Committee Scandinavian Society of Anaesthesiology and Intensive Care Medicine). Pre-operative fasting guidelines: an update. Acta Anaesthesiol Scand 2005;49:1041–7
4. Smith I, Kranke P, Murat I, Smith A, O'Sullivan G, Søreide E, Spies C, in't Veld B; European Society of Anaesthesiology. Perioperative fasting in adults and children: guidelines from the European Society of Anaesthesiology. Eur J Anaesthesiol 2011;28:556–69
5. Nygren J, Thorell A, Jacobsson H, Larsson S, Schnell PO, Hylén L, Ljungqvist O. Preoperative gastric emptying. Effects of anxiety and oral carbohydrate administration. Ann Surg 1995;222:728–34
6. Brady M, Kinn S, Stuart P. Preoperative fasting for adults to prevent perioperative complications. Cochrane Database Syst Rev 2003;4:CD004423
7. Hausel J, Nygren J, Lagerkranser M, Hellström PM, Hammarqvist F, Almström C, Lindh A, Thorell A, Ljungqvist

O. A carbohydrate-rich drink reduces preoperative discomfort in elective surgery patients. Anesth Analg 2001;93:1344–50
8. Nygren J. The metabolic effects of fasting and surgery. Best Pract Res Clin Anaesthesiol 2006;20:429–38
9. Kratzing C. Pre-operative nutrition and carbohydrate loading. Proc Nutr Soc 2011;70:311–5
10. Lobo DN, Hendry PO, Rodrigues G, Marciani L, Totman JJ, Wright JW, Preston T, Gowland P, Spiller RC, Fearon KC. Gastric emptying of three liquid oral preoperative metabolic preconditioning regimens measured by magnetic resonance imaging in healthy adult volunteers: a randomised double-blind, crossover study. Clin Nutr 2009;28:636–41
11. Noblett SE, Watson DS, Huong H, Davison B, Hainsworth PJ, Horgan AF. Pre-operative oral carbohydrate loading in colorectal surgery: a randomized controlled trial. Colorectal Dis 2006;8:563–9
12. Breuer JP, von Dossow V, von Heymann C, Griesbach M, von Schickfus M, Mackh E, Hacker C, Elgeti U, Konertz W, Wernecke KD, Spies CD. Preoperative oral carbohydrate administration to ASA III-IV patients undergoing elective cardiac surgery. Anesth Analg 2006;103:1099–108
13. Yuill KA, Richardson RA, Davidson HI, Garden OJ, Parks RW. The administration of an oral carbohydrate-containing fluid prior to major elective upper-gastrointestinal surgery preserves skeletal muscle mass postoperatively–a randomised clinical trial. Clin Nutr 2005;24:32–7
14. Hendry PO, Balfour A, Potter MA, Mander BJ, Bartolo DC, Anderson DN, Fearon KC. Preoperative conditioning with oral carbohydrate loading and oral nutritional supplements can be combined with mechanical bowel preparation prior to elective colorectal resection. Colorectal Dis 2008;10:907–10
15. Itou K, Fukuyama T, Sasabuchi Y, Yasuda H, Suzuki N, Hinenoya H, Kim C, Sanui M, Taniguchi H, Miyao H, Seo N, Takeuchi M, Iwao Y, Sakamoto A, Fujita Y, Suzuki T. Safety and efficacy of oral rehydration therapy until 2 h before surgery: a multicenter randomized controlled trial. J Anesth 2012;26:20–7
16. Fearon KC, Ljungqvist O, Von Meyenfeldt M, Revhaug A, Dejong CH, Lassen K, Nygren J, Hausel J, Soop M, Andersen J, Kehlet H. Enhanced recovery after surgery: a consensus review of clinical care for patients undergoing colonic resection. Clin Nutr 2005;24:466–77
17. Yatabe J, Saito F, Ishida I, Sato A, Hoshi M, Suzuki K, Kameda T, Ueno S, Yatabe MS, Watanabe T, Sanada H. Lower plasma arginine in enteral tube-fed patients with pressure ulcer and improved pressure ulcer healing after arginine supplementation by Arginaid Water. J Nutr Health Aging 2011;15:282–6
18. Okamoto Y, Okano K, Izuishi K, Usuki H, Wakabayashi H, Suzuki Y. Attenuation of the systemic inflammatory response and infectious complications after gastrectomy with preoperative oral arginine and omega-3 fatty acids supplemented immunonutrition. World J Surg 2009;33:1815–21
19. Moore FA, Moore EE, Kudsk KA, Brown RO, Bower RH, Koruda MJ, Baker CC, Barbul A. Clinical benefits of an immune-enhancing diet for early postinjury enteral feeding. J Trauma 1994;37:607–15
20. Cordova Martinez A, Escanero Marcen JF. Changes in serum trace elements after surgery: value of copper and zinc in predicting post-operative fatigue. J Int Med Res 1992;20:12–9
21. Unoshima M, Nishizono A, Takita-Sonoda Y, Iwasaka H, Noguchi T. Effects of zinc acetate on splenocytes of endotoxemic mice: enhanced immune response, reduced apoptosis, and increased expression of heat shock protein 70. J Lab Clin Med 2001;137:28–37
22. Sakurai Y, Uchida M, Aiba J, Mimura F, Yamaguchi M. Safe practice of oral rehydration therapy by oral rehydration solution and carbohydrate loading–evaluation by non-invasive gastric echo examination. Masui 2011;60:790–8
23. Schwizer W, Maecke H, Fried M. Measurement of gastric emptying by magnetic resonance imaging in humans. Gastroenterology 1992;103:369–76
24. Feinle C, Kunz P, Boesiger P, Fried M, Schwizer W. Scintigraphic validation of a magnetic resonance imaging method to study gastric emptying of a solid meal in humans. Gut 1999;44:106–11
25. Wright J, Evans D, Gowland P, Mansfield P. Validation of antroduodenal motility measurements made by echo-planar magnetic resonance imaging. Neurogastroenterol Motil 1999;11:19–25
26. Horowitz M, Dent J, Fraser R, Sun W, Hebbard G. Role and integration of mechanisms controlling gastric emptying. Dig Dis Sci 1994;39:7S–13S
27. Vist GE, Maughan RJ. The effect of osmolality and carbohydrate content on the rate of gastric emptying of liquids in man. J Physiol 1995;486:523–31
28. Taylor IL, Byrne WJ, Christie DL, Ament ME, Walsh JH. Effect of individual l-amino acids on gastric acid secretion and serum gastrin and pancreatic polypeptide release in humans. Gastroenterology 1982;83:273–8
29. Kidd M, Modlin IM, Tang LH. Gastrin and the enterochromaffin-like cell: an acid update. Dig Surg 1998;15:209–17
30. Wendland BE, Arbus GS. Oral fluid therapy: sodium and potassium content and osmolality of some commercial "clear" soups, juices and beverages. Can Med Assoc J 1979;121:564–6, 568, 571
31. Katz H, Butler P, Homan M, Zerman A, Caumo A, Cobelli C, Rizza R. Hepatic and extrahepatic insulin action in humans: measurement in the absence of non-steady-state error. Am J Physiol 1993;264:E561–6
32. Datz FL, Christian PE, Moore J. Gender-related differences in gastric emptying. J Nucl Med 1987;28:1204–7
33. Degen LP, Phillips SF. Variability of gastrointestinal transit in healthy women and men. Gut 1996;39:299–305
34. Goo RH, Moore JG, Greenberg E, Alazraki NP. Circadian variation in gastric emptying of meals in humans. Gastroenterology 1987;93:515–8
35. Van Cauter E, Polonsky KS, Scheen AJ. Roles of circadian rhythmicity and sleep in human glucose regulation. Endocr Rev 1997;18:716–38

Journal of Immunological Methods 413 (2014) 1–11

Contents lists available at ScienceDirect

Journal of Immunological Methods

journal homepage: www.elsevier.com/locate/jim

Research paper

A standardized blood test for the routine clinical diagnosis of impaired GM-CSF signaling using flow cytometry

Yoshiomi Kusakabe [a], Kanji Uchida [a,b,*], Takahiro Hiruma [a], Yoko Suzuki [a], Tokie Totsu [a], Takuji Suzuki [b], Brenna C. Carey [b], Yoshitsugu Yamada [a], Bruce C. Trapnell [b,c,*,1]

[a] The University of Tokyo Graduate School of Medicine, Tokyo, Japan
[b] Children's Hospital Research Foundation, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, USA
[c] University of Cincinnati College of Medicine, Cincinnati, OH, USA

ARTICLE INFO

Article history:
Received 27 May 2014
Received in revised form 2 July 2014
Accepted 21 July 2014
Available online 26 July 2014

Keywords:
Pulmonary alveolar proteinosis
CD11b
Neutrophil
Granulocyte/macrophage-colony stimulating factor
Flow cytometry
Diagnosis

ABSTRACT

Impaired signaling by granulocyte/macrophage-colony stimulating factor (GM-CSF) drives the pathogenesis of two diseases (autoimmune and hereditary pulmonary alveolar proteinosis (PAP)) representing over ninety percent of patients who develop PAP syndrome but not a broad spectrum of diseases that cause PAP by other mechanisms. We previously exploited the ability of GM-CSF to rapidly increase cell-surface CD11b levels on neutrophils ($CD11b_{Surface}$) to establish the CD11b stimulation index (CD11b-SI), a test enabling the clinical research diagnosis of impaired GM-CSF signaling based on measuring $CD11b_{Surface}$ by flow cytometry using fresh, heparinized blood. (CD11b-SI is defined as GM-CSF-stimulated- $CD11b_{Surface}$ minus unstimulated $CD11b_{Surface}$ divided by unstimulated $CD11b_{Surface}$ multiplied by 100.) Notwithstanding important and unique diagnostic utility, the test is sensitive to experimental conditions that can affect test performance. The present study was undertaken to optimize and standardize CD11b-SI test for detecting impaired GM-CSF signaling in heparinized human blood specimens from PAP patients. Results demonstrated the test was sensitive to choice of anticoagulant, pretesting incubation on ice, a delay between phlebotomy and test performance of more than one hour, and the concentration GM-CSF used to stimulate blood. The standardized CD11b-SI test reliably distinguished blood specimens from autoimmune PAP patients with impaired GM-CSF signaling from those of health people with normal signaling. Intra-subject differences were smaller than inter-subject differences in repeated measures. Receiver operating characteristic curve analysis identified a CD11b-SI test result of 112 as the optimal cut off threshold for diagnosis of impaired GM-CSF signaling in autoimmune PAP for which the sensitivity and specificity were both 100%. These results support the use of this standardized CD11b-SI for routine clinical identification of impaired GM-CSF signaling in patients with autoimmune PAP. The CD11b-SI may also have utility in clinical trials of novel therapeutic strategies targeting reduction in GM-CSF bioactivity now under evaluation for multiple common autoimmune and inflammatory disorders.

1. Introduction

Granulocyte/macrophage-colony-stimulating factor (GM-CSF) is a cytokine with pleiotropic effects on myeloid cells including stimulation of the survival, proliferation, and differentiation myeloid progenitors, as well as augmentation ("priming") of host defense functions of mature macrophages and neutrophils (Lieschke and Burgess, 1992; Condliffe et al., 1998).

* Corresponding authors at: Divisions of Pulmonary Biology and Medicine, Cincinnati Children's Hospital Medical Center, Room 4029, CCRF, 3333 Burnet Avenue, Cincinnati, OH 45229-3039, USA. Tel.: +1 513 636 6361; fax: +1 513 636 3723.
E-mail addresses: Y-KUSAKABE@NIFTY.COM (Y. Kusakabe), uchidak-ane@h.u-tokyo.ac.jp (K. Uchida), hirumat-eme@h.u-tokyo.ac.jp (T. Hiruma), yoko.suzuki@ruri.waseda.jp (Y. Suzuki), himehime0610@gmail.com (T. Totsu), Takuji.Suzuki@cchmc.org (T. Suzuki), Brenna.Carey@cchmc.org (B.C. Carey), yamaday-ane@h.u-tokyo.ac.jp (Y. Yamada), Bruce.Trapnell@cchmc.org (B.C. Trapnell).
[1] Designated to communicate with the Journal Editor.

http://dx.doi.org/10.1016/j.jim.2014.07.009
0022-1759/

Although normally present at low or undetectable levels in vivo (Uchida et al., 2009), GM-CSF is critical to the normal functioning of blood neutrophils (Uchida et al., 2007) and alveolar macrophages (Trapnell and Whitsett, 2002). In humans, disruption of GM-CSF signaling by high levels of neutralizing GM-CSF autoantibodies is associated with the development of autoimmune pulmonary alveolar proteinosis (autoimmune PAP), a rare lung disease characterized by surfactant accumulation, respiratory failure, and increased infections (Kitamura et al., 1999; Trapnell et al., 2003; Uchida et al., 2004, 2007). However, GM-CSF autoantibodies are present at low levels in healthy people (Uchida et al., 2009) and levels do not correlate with disease severity in PAP patients (Seymour et al., 2003). This apparent paradox is rectified by the concept of a critical threshold of GM-CSF autoantibodies above which GM-CSF-dependent functions (like surfactant clearance by alveolar macrophages) are reduced to zero regardless of the magnitude of the increase above the critical threshold (Bendtzen et al., 2007). Disruption of GM-CSF receptor function by recessive *CSF2RA* or *CSF2RB* mutations causes hereditary PAP, which is histologically indistinguishable from autoimmune PAP (Martinez-Moczygemba et al., 2008; Suzuki et al., 2008, 2010). PAP also occurs in a heterogeneous group of diseases either as a consequence of an underlying clinical condition presumably affecting the alveolar macrophage function (secondary PAP) (Ishii et al., 2009) or by mutations in genes involved in surfactant production (e.g., *SFTPB, SFTPC, ABCA3, TTF1*) (congenital PAP, and PAP associated with interstitial lung disease)(Nogee, 2010; Whitsett et al., 2004). In genetically modified mice, GM-CSF deficiency causes PAP (Dranoff et al., 1994) while GM-CSF overexpression causes a syndrome of macrophage accumulation, tissue damage, and death (Lang et al., 1987). Increased GM-CSF bioactivity has been implicated in the pathogenesis of rheumatoid arthritis, multiple sclerosis, and other inflammatory and autoimmune diseases and evaluation of GM-CSF antagonist therapy is underway in human clinical trials for multiple clinical indications (Hamilton, 2008). These observations indicate that GM-CSF bioactivity is tightly controlled in healthy people, that loss of tight control is involved in the pathogenesis of multiple diseases and suggest the utility of a clinical test to measure impaired GM-CSF signaling in humans.

CD11b is a cell-adhesion molecule normally present in resting neutrophils within pre-formed granules that is translocated to the cell surface upon neutrophil activation (Graves et al., 1992) including after exposure to an increased concentration of GM-CSF (Condliffe et al., 1998). Previously, we exploited this endogenous priming mechanism in developing the CD11b stimulation index (CD11b-SI), a test to measure impaired GM-CSF signaling in human blood specimens (Uchida et al., 2007). In this assay, fresh, heparinized whole blood is incubated with and without GM-CSF and the mean fluorescence of CD11b on $CD16^{Hi}$ leukocytes is measured by flow cytometry to determine the level of cell-surface CD11b on neutrophils ($CD11b_{Surface}$). The GM-CSF stimulated increase in $CD11b_{Surface}$ (i.e., the stimulation index or SI), is large and readily detected in blood specimens from healthy individuals and zero or severely reduced in patients with autoimmune PAP or hereditary PAP (Uchida et al., 2007, 2009; Suzuki et al., 2008). In the present study, we evaluated and optimized the experimental conditions of the CD11b-SI assay and then validated the test using clinical specimens from patients previously diagnosed with autoimmune PAP and from healthy people.

2. Methods

2.1. Participants

The institutional review board of the Cincinnati Children's Hospital Medical Center (CCHMC) and the University of Tokyo Graduate School of Medicine approved the study. All participants or their legal guardians gave written informed consent, and minors gave assent. Participates included 10 individuals referred for evaluation or treatment of autoimmune PAP diagnosed based on clinical and radiographic findings; an open lung biopsy, transbronchial lung biopsy, or cytologic analysis of bronchoalveolar lavage cells and fluid; and a positive GMAb test performed as described (Uchida et al., 2014). We also studied 34 healthy control subjects who were nonsmokers with no history of major illness and symptom-free at the time of enrollment in the study. Data for some individuals were previously reported (Uchida et al., 2007, 2009; Han et al., 2009).

2.2. Reagents

Primary antibodies included FITC- or PE-conjugated anti-human CD11b, PE-conjugated anti-human CD16 (Miltenyi Biotec K.K., Tokyo, Japan); FITC-rat IgG (isotype control) (abcam, Cambridge, UK); anti-human CD11b monoclonal (BioLegend, San Diego, CA); anti-human phospho-STAT5 (Millipore, Billerica, MA). Secondary detection antibody (anti-rat IgG-HRP(HAF005)) was from R&D systems (Minneapolis, MN) and anti-mouse IgG-HRP was from GE Healthcare (Little Challfont Buckinghamshire, UK). HRP-conjugated anti-actin antibody was from Santa Cruz Biotechnology (Dallas, TX). *E. coli*-derived recombinant human GM-CSF was from ATGen (Seongnam, South Korea). Recombinant cytokines, and other proteins included G-CSF, C5a, Interleukin (IL)-6, IL-8, IL-10, Interferon (IFN)-β, IFN-γ (all from Wako, Osaka, Japan).

2.3. Flow cytometry

Evaluation of neutrophils by flow cytometry was performed as previously described (Uchida et al., 2007). Briefly, 200 μL of whole blood in a 1.5 ml polypropylene tube was incubated (37 °C, 30 minutes) with recombinant human GM-CSF (10 ng/mL) or other cytokines / chemokines, placed on ice immediately. An aliquot (50 μL) of each was mixed with 50 μL of ice-cold PBS containing FITC-conjugated anti-human CD11b antibody (4 μL, undiluted) and PE-conjugated anti-human CD16 antibody (4 μL, undiluted) in a polystyrene round-bottom tube (BD Bioscience, San Jose, CA) and incubated (4 °C, 30 min, in darkness). After adding 2 mL of FACS lysing solution (BD Biosciences, San Jose, CA), incubation (room temp, 10 min), tubes were centrifuged (300 ×g, room temp, 5 min). The cell pellets were re-suspended in 200 μL of PBS and stored in 4 °C in the dark until evaluation by flow cytometry. The amount of cell-surface CD11b on neutrophils ($[CD11b_{Surface}]$) was measured by flow cytometry as mean

fluorescent intensity of FITC on $CD16^{Hi}$ cells defined by gating. The CD11b stimulation index (CD11b-SI) was calculated using the following equation

$$CD11b\text{-}SI = ([CD11b_{Surface}]_{+GM\text{-}CSF} - [CD11b_{Surface}]_{No\ GM\text{-}CSF}) \times 100 / [CD11b_{Surface}]_{No\ GM\text{-}CSF}$$

where $[CD11b_{Surface}]_{+GM\text{-}CSF}$ represents $[CD11b_{Surface}]$ after incubation with GM-CSF and $[CD11b_{Surface}]_{No\ GM\text{-}CSF}$ represents $[CD11b_{Surface}]$ after incubation without GM-CSF. In some experiments, cytoplasmic CD11b was stained with PE-CD11b (4 μl, undiluted) using the Intracellular Cytokine Staining Kit (eBioscience, San Diego, CA) as per manufacturer's instructions.

2.4. Western blotting

Heparinized whole blood was incubated with or without GM-CSF (10 ng/mL, 37 °C, 15 min) and red blood cells were lysed with lysing buffer (BD Pharmlyse™, BD Biosciences, San Jose, CA). After washing with ice-cold PBS, cells were extracted with protein extraction buffer (M-PER® #78501) containing protease inhibitor cocktail (0.5% v/v), phosphatase inhibitor cocktail (1% v/v) (all from Thermo Scientific, Rockford, IL), and EDTA (5 mM). Protein extracts were suspended in sample loading buffer, layered onto AnykD gradient gels (Bio-rad, Hercules, CA), separated by gel electrophoresis (100 V, 150 minutes) and transferred (Transblot™, Bio-rad, Hercules, CA) onto PVDF membranes (Immobilon, Merck Millipore, Billerica, MA), per manufacturer's instructions. Membranes were incubated with either murine anti-CD11b monoclonal antibody (10 μl from the vial diluted in 500 μl of immunoblotting buffer (Uchida 2009) (BioLegend, San Diego, CA) or murine anti-phospho- STAT5 antibody (5 μl from the vial diluted in 1000 μl of immunoblotting buffer (Millipore, Billerica, MA)) and detection was done using ECL plus™ (GE healthcare, Little Chalfont, UK). Band intensity of CD11b was quantified by Image Quant TL Analysis Toolbox Ver 7.0 (GE Healthcare, Pittsburgh, PA).

2.5. Statistical analysis

Numerical data were evaluated for a normal distribution using the Kolmogorov-Smirnov test and for equal variance using the Levene median test; parametric data are presented as mean (±SD) and nonparametric data are presented as median and interquartile range (IQR). Statistical comparisons of parametric data were made with Student's t-test for two-group comparisons and with one-way analysis of variance with post hoc analysis according to the Holm-Sidak method for multiple-group comparisons or Dunnett's method for multiple comparisons versus control. Nonparametric data were compared with the use of the Mann-Whitney rank-sum test. Intra-individual percent variation was calculated as the average, for all measured values from each person, of the value minus the mean of all values for an individual divided by the mean and multiplied by 100. Inter-individual percent variation was calculated as the average, for all individuals, of the final value for each individual minus the mean for all individuals divided by the mean for all individuals and multiplied by 100. Receiver operating characteristic (ROC) curve and all other statistical analyses were done using SigmaPlot software, version 12 (Systat Software, SanJose, CA). P values less than 0.05 were considered to indicate statistical significance and are denoted by asterisks (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). All experiments were repeated at least twice with similar results.

3. Results

3.1. Optimization of CD11b-SI components and procedure

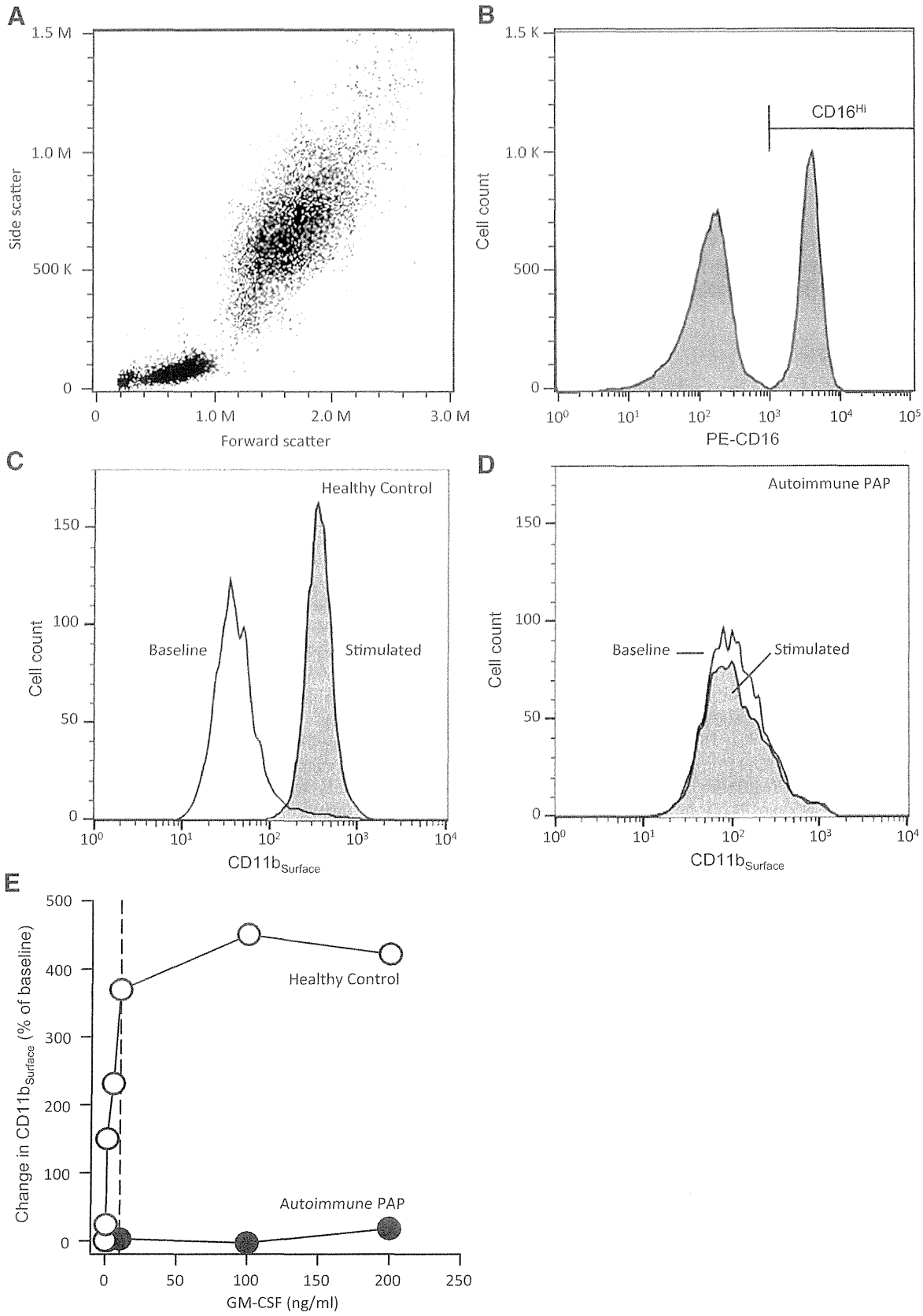
Neutrophils in fresh heparinized whole blood identified by $CD16^{Hi}$ immunostaining (Fig. 1A and B) had detectable levels of cell-surface CD11b ($CD11b_{Surface}$) at baseline (i.e., without stimulation) in both healthy individuals (1C) and autoimmune PAP patients (1D). GM-CSF stimulated a significant increase in $CD11b_{Surface}$ in blood from healthy individuals (Figure C) but not in blood from autoimmune PAP patients (Figure D). The change from baseline $CD1b_{Surface}$ increased with GM-CSF concentration and reached saturation at ~10 ng/mL in healthy people and was severely blunted with little increase in patients with autoimmune PAP (Fig. 1E). To exploit this GM-CSF-stimulated change in $CD11b_{Surface}$ as a measure of impaired GM-CSF signaling in clinical samples, we first optimized experimental conditions to establish a standardized test to measure impaired GM-CSF signaling in whole blood.

The effect of anticoagulant on $CD11b_{Surface}$ was evaluated in fresh whole blood collected into either EDTA- or heparin-containing phlebotomy specimen tubes. Baseline, un-stimulated $CD11b_{Surface}$ was not different among blood specimens collected using EDTA or heparin ($P = 0.174$). In contrast, the GM-CSF-stimulated increase in $CD11b_{Surface}$ was smaller when EDTA was used for phlebotomy compared to samples collected in heparin (Fig. 2).

Blood is frequently collected at room temperature and maintained / evaluated at room temperature, on ice, or at 37 °C, depending on assay requirements. Therefore, we evaluated the effect of temperature on baseline $CD11b_{Surface}$ using fresh whole blood collected at room temperature (~25 °C), and then maintained briefly at different combinations of these three temperatures. When blood was incubated at 25 °C and then at 37 °C (30 min each) or maintained at 37 °C for 60 min, baseline $CD11b_{Surface}$ was unaffected (Fig. 3). However, when blood was incubated at 0 °C and then at 37 °C (30 min each), $CD11b_{Surface}$ increased by 140% (Fig. 3). Consequently, subsequent studies were conducted with blood maintained at room temperature before evaluation.

Next we evaluated potential effects of time before blood specimen evaluation on baseline $CD11b_{Surface}$. The ability of neutrophils to exhibit an increase in $CD11b_{Surface}$ after incubation of heparinized whole blood with 10 ng/mL GM-CSF compared to baseline $CD11b_{Surface}$ (henceforth, change in $CD11b_{Surface}$ (%)) was robust immediately after phlebotomy but markedly diminished after 1 day and declined further thereafter (Fig. 4). In contrast, when blood was stimulated and then stained and fixed immediately after phlebotomy, flow cytometric analysis could be performed for up to seven days with little to no effect on the change in $CD11b_{Surface}$ (Fig. 4).

To further explore the effects of time and temperature on the change in $CD11b_{Surface}$, we evaluated the effects of a short-term delay between phlebotomy and testing for freshly isolated, heparinized whole blood stored at various temperatures. $CD11b_{Surface}$ remained constant for up to 6 hours when


A
1.5 M
1.0 M
500 K
0
Side scatter
0
1.0 M
2.0 M
3.0 M
Forward scatter
B
1.5 K
1.0 K
500
0
Cell count
CD16Hi
10^0
10^1
10^2
10^3
10^4
10^5
PE-CD16
C
150
100
50
0
Cell count
Healthy Control
Baseline
Stimulated
10^0
10^1
10^2
10^3
10^4
CD11bSurface
D
150
100
50
0
Cell count
Autoimmune PAP
Baseline
Stimulated
10^0
10^1
10^2
10^3
10^4
CD11bSurface
E
500
400
300
200
100
0
Change in CD11bSurface (% of baseline)
Healthy Control
Autoimmune PAP
0
50
100
150
200
250
GM-CSF (ng/ml)

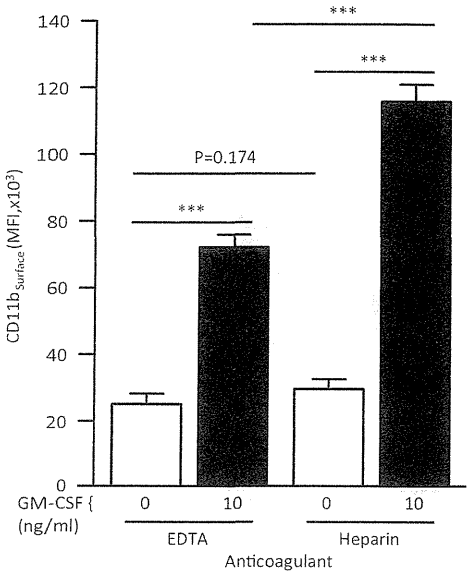


Fig. 2. Effect of anticoagulant on baseline and GM-CSF-stimulated CD11b$_{Surface}$. Fresh blood was collected from healthy people into ethylenediaminetetraacetic acid (EDTA) – or sodium heparin-containing phlebotomy tubes and the mean fluorescence intensity (MFI) of CD11b on CD16^{Hi}-gated neutrophils (CD11b$_{Surface}$) was measured after stimulation with 10 ng/ml GM-CSF using the CD11b-SI test as described in the Methods. Bars represent the mean ± SD for n = 3 per condition. Comparisons were made by ANOVA with Dunnett's test; *** = P < 0.001.

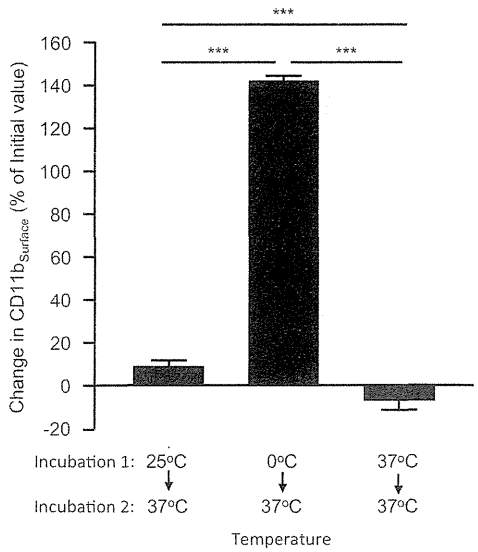


Fig. 3. Effect of change in the temperature at which blood was maintained before testing on the CD11b$_{Surface}$. Fresh, heparinized whole blood was collected from healthy people by phlebotomy and immediately incubated at 0 °C, 25 °C, or 37 °C for 30 min (incubation period 1) followed immediately by incubation at 37 °C for 30 min (incubation period 2) and then CD11b$_{Surface}$ was measured as described in the Methods. For each sample, the CD11b$_{Surface}$ at the end of incubation period 2 was subtracted from the CD11b$_{Surface}$ at the end of incubation period 1, divided by the CD11b$_{Surface}$ at the end of incubation period 1 and multiplied by 100 percent. Positive values indicate an increase in CD11b$_{Surface}$ after incubation period 2 compared to after incubation period 1. Bars represent the mean ± SD for n = 3 per condition. Comparisons were made by ANOVA with Dunnett's test; *** = P < 0.001.

blood was kept on ice but increased progressively when blood was kept at room temperature (and even faster when blood was kept at 37 °C) during this time period (Fig. 5A).

In parallel, the change in CD11b$_{Surface}$ declined over the time period for blood maintained at 25 °C (Fig. 5B).

To better understand the mechanism of the GM-CSF-stimulated increase in CD11b$_{Surface}$, the spatial distribution of CD11b within neutrophils (i.e., in the cytoplasm or on the cell-surface) was evaluated before and after GM-CSF stimulation using several methods. Simultaneous detection of CD11b$_{Surface}$ and cytoplasmic CD11b (CD11b$_{Cytosol}$) in cells double-immunostained with FITC-CD11b or PE-CD11b, respectively (Fig. 6A and B), revealed that CD11b$_{Surface}$ increased by 150 % with GM-CSF stimulation (Fig. 6C). In contrast, CD11b$_{Cytosol}$ decreased by ~11% and CD11b$_{Total}$ was not different (Fig. 6C). CD11bTotal was also unchanged by GM-CSF-stimulation when both CD11b$_{Surface}$ and CD11b$_{Cytosol}$ were measured together using the same detection antibody/chromophore (FITC-anti-human CD11b) (Fig. 6D) or when measured by western blotting (Fig. 6D). Finally, CD11b$_{Surface}$ gradually increased while CD11b$_{Cytosol}$ gradually decreased in fresh whole blood maintained at 25 °C for 6 hours (Fig. 6E). Together, these results indicate that GM-CSF stimulates pre-formed CD11b located within the neutrophil to translocate to the cell surface after stimulation.

The specificity of the change in CD11b$_{Surface}$ was evaluated by comparing the change in CD11b$_{Surface}$ stimulated by GM-CSF to that caused by a wide range of pro- and anti-inflammatory mediators. Compared to baseline, GM-CSF stimulated a greater increase in CD11b$_{Surface}$ than did IL-8, IL-6, G-CSF, interferon (IFN)-β, IL-10, IFN-γ, and complement fragment 5a (Fig. 7).

Taken together, these results indicate that GM-CSF specifically stimulates neutrophils causing a robust and rapid translocation of pre-formed, intracytoplasmic CD11b to the cell surface.

3.2. CD11b-SI test performance

Next, we evaluated the ability of the standardized test to identify impaired GM-CSF signaling in whole blood in patients with autoimmune PAP in whom GM-CSF signaling is known to be impaired by high concentrations of neutralizing GM-CSF autoantibodies and in healthy people in whom GM-CSF signaling in blood leukocytes is present and readily detectable (Uchida et al., 2009). The baseline level of CD11b$_{Surface}$ in autoimmune PAP patients was higher than in healthy individuals (170.2 ± 75.4, 101.4 ± 63.2, n = 10, 25, respectively, p = 0.010; not shown). At the standardized concentration for testing (10 ng/ml), GM-CSF stimulated a mean increase from baseline in CD11b$_{Surface}$ of ~450% in 22 healthy people but no increase in 5 patients with autoimmune PAP (Fig. 8). The difference in GM-CSF-stimulated CD11b$_{Surface}$

Fig. 1. Evaluation of CD11b$_{Surface}$ on the neutrophil in the whole blood: an assay to measure the GM-CSF-stimulated increase in cell-surface CD11b on neutrophils in whole blood. A. Representative leukocyte cytogram. Whole blood was processed and evaluated by flow cytometry as described in the Methods. B. Identification of neutrophils by gating on CD16. Immunostained leukocytes were gated for phycoerythrin (PE)-fluorescence to identify neutrophils as a distinct CD16^{HI} population. C-D. Quantification of neutrophil cell-surface CD11b (CD11b$_{Surface}$) for a healthy control (C) and a patient with autoimmune PAP (D). Representative histogram of the fluorescence intensity in neutrophils from healthy control. open area – no GM-CSF stimulation; filled area – after GM-CSF stimulation. E. Percent change in CD11b$_{Surface}$ for healthy individual (HC) and patient with autoimmune pulmonary alveolar proteinosis (PAP).

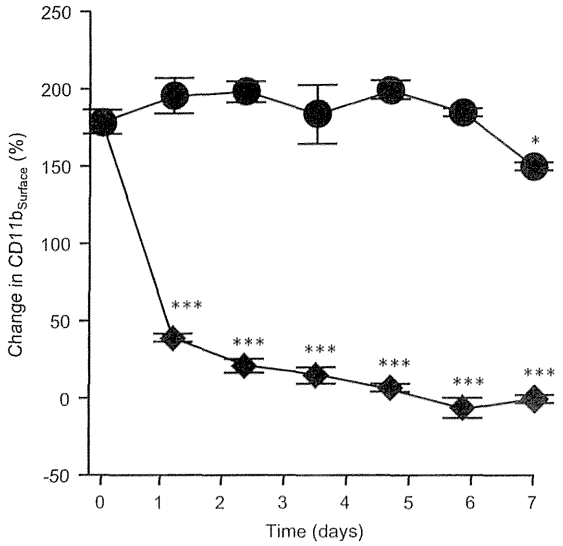


Fig. 4. Effect of time between GM-CSF stimulation/fixation and evaluation by flow cytometry on the change in $CD11b_{Surface}$. Fresh, heparinized blood was collected from healthy people, incubated with or without GM-CSF within 1 hour of phlebotomy, staining with antibodies to $CD11b_{Surface}$, and fixed (closed symbols) or stored at room temperature for various times (open symbols) before stimulation, immunostaining and analysis by flow cytometry as described in the Methods. Symbols represent the mean ± SD for n = 3 per point. Comparisons to the corresponding value on day zero were made by ANOVA with Dunnett's test; * = $P < 0.05$, *** = $P < 0.001$.

between healthy people and autoimmune PAP patients was also observed at lower (1 ng/ml) and higher (100 ng/ml) GM-CSF stimulating concentrations, although there was a small increase in the latter (that remained significantly different from healthy controls) (Fig. 8).

We determined CD11b stimulation index or CD11b-SI as change in $CD11b_{Surface}$ at 10 ng/mL of GM-CSF to maximize discrepancies between healthy controls and patients with PAP.

The precision of the CD11b-SI test for measuring impaired GM-CSF signaling in clinical blood specimens was evaluated by measuring the inter-subject and intra-subject variation in fresh heparinized whole blood from nine healthy people (Fig. 9A). The coefficient of variation for repeated measures in the same subject was 5.3 ± 3.5%, which is less than 15% in accordance with FDA guidance criteria for assay precision (Anonymous, 2001) (Fig. 9B). Further, the coefficient of variation for measurements within subject measurements was less than that for measurements between subjects (Fig. 9B). These data indicate the CD11b-SI test is reliable and can be used to measure impaired GM-CSF signaling in clinical blood samples.

3.3. Diagnostic cutoff of the CD11b-SI

To further support the diagnostic use of the CD11b-SI test to diagnose impaired GM-CSF signaling in human clinical specimens, we measured the CD11b-SI in people previously diagnosed with autoimmune PAP and in healthy, asymptomatic people. The CD11b-SI in patients with autoimmune PAP (3.61 [-7.97 – 10.95]; n = 10) was markedly lower than in healthy people (321 [195 - 524]; n = 34) (Fig. 10A). There was a clear separation between the high values in healthy people and the low values in autoimmune PAP patients (Fig. 10B).

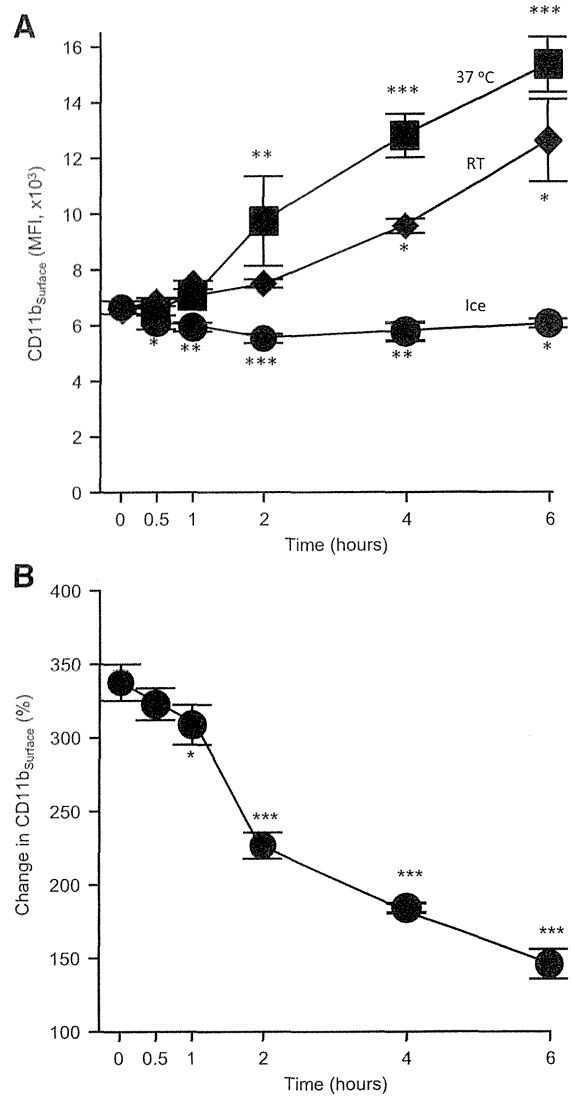


Fig. 5. Effects of temperature of blood after phlebotomy and delay in analysis on change in $CD11b_{Surface}$. A. Effect of temperature on $CD11b_{Surface}$ expression levels. Fresh, heparinized blood was collected and maintained at the indicated temperatures and then CD11b expression levels were evaluated as described in the Methods. B. Effect of a short delay prior to analysis on the change in $CD11b_{Surface}$ stimulated with 10 ng/mL of GM-CSF. Fresh, heparinized blood was kept at 25 °C for various times before evaluation by the change in $CD11b_{Surface}$ as described in the Methods. Symbols represent the mean ± SD for n = 3 per point. Comparisons to the corresponding value at zero hours were made by ANOVA with Dunnett's test; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$.

Receiver operating characteristic (ROC) curve analysis for these results demonstrated the sensitivity and specificity to be 100% (Fig. 10C) and identified an optimal cut off threshold value for the difference between normal and abnormal CD11b-SI values (i.e., in healthy people and patients with autoimmune PAP, respectively) to be 112 (Fig. 10D).

4. Discussion

In this study, we optimized the experimental conditions of the CD11b-SI test (Uchida et al., 2007) and then evaluated the

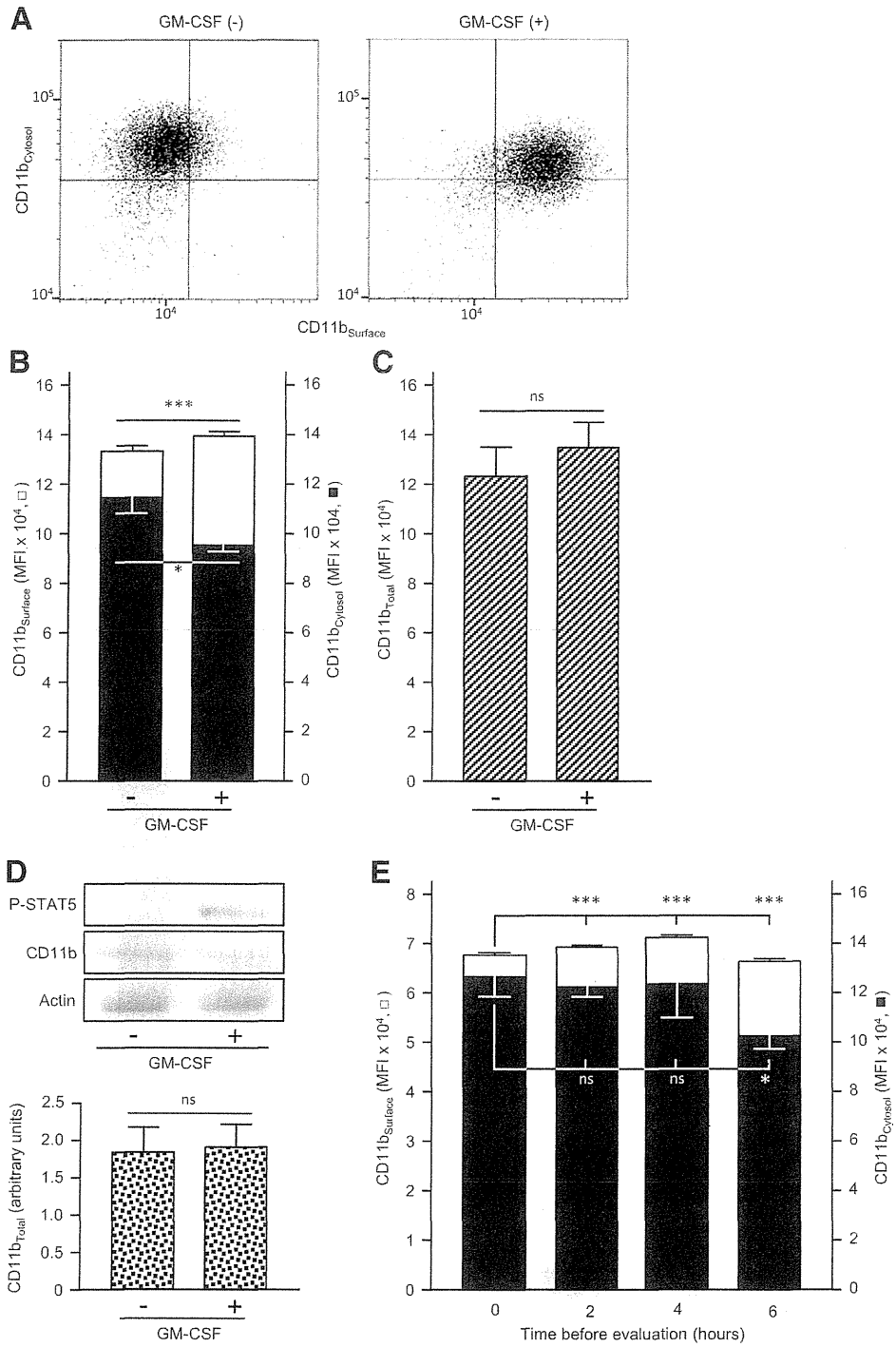


Fig. 6. GM-CSF-stimulated translocation of CD11b to the neutrophil cell-surface. A,B. Fresh, heparinized blood from healthy people was evaluated for the level of CD11b except that cells were first immunostained with FITC-anti-human CD11b to measure $CD11b_{Surface}$ (open bars), washed, permeabilized and stained with PE-anti-human CD11b to measure cytosolic $CD11b_{Cytosol}$ (filled bars) as described in the Methods. A. Representative cytograms of neutrophils stained to show $CD11b_{Surface}$ (horizontal axis) and $CD11b_{Cytosol}$ (vertical axis) with or without GM-CSF stimulation (indicated). B. Quantification of $CD11b_{Surface}$ (open bars) and $CD11b_{Cytosol}$ (closed bars) on neutrophils. Bars represent the mean ± SD for n = 3 per condition. C. Both $CD11b_{Surface}$ and $CD11b_{Cytosol}$ were evaluated with methods described above (legends to Panel A,B) with FITC-anti-human CD11b for both to measure total neutrophil CD11b levels. Bars represent the mean ± SD for n = 3 per condition. D. Quantification of total CD11b by western blotting. Proteins of white blood cell in the whole blood incubated with or without GM-CSF (10 ng/mL) was evaluated by gel electrophoresis and western blotting to quantify CD11b, phosphorylated-STAT5 (as a GM-CSF stimulation control), or actin (as a loading control), and CD11b band intensity quantified by densitometry as described in the Methods. Bars represent mean ± SD for n = 4 determinations per condition. E. Cytosolic CD11b translocation to the neutrophil cell surface with time. Fresh, heparinized blood from healthy people was incubated at room temperature for various times before differential immunostaining to quantify $CD11b_{Surface}$ (open bars) and $CD11b_{Cytosol}$ (filled bars) as described above (legend to Panel A). Bars represent the mean ± SD of n = 3 per condition. Comparisons were made by Student's t test (A-C) or ANOVA (D); * = $P < 0.05$, *** = $P < 0.001$.

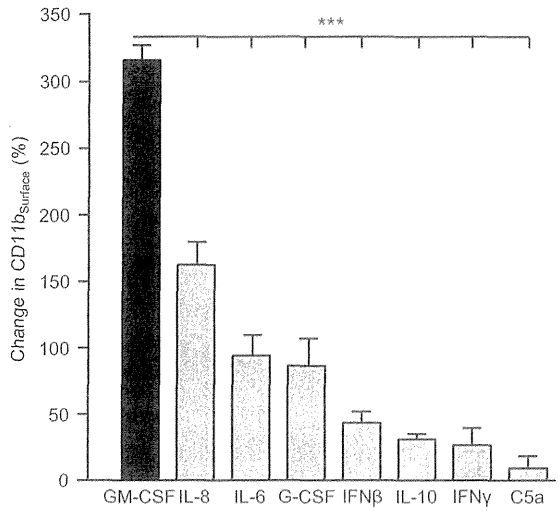


Fig. 7. Capacity of GM-CSF and other neutrophil activators to increase $CD11b_{Surface}$. Fresh, heparinized blood from healthy people was incubated without or with GM-CSF or other cytokines, chemokines, and inflammatory molecules including interleukin 8 (IL-8), IL-6, granulocyte-colony stimulating factor (G-CSF), interferon β (IFNβ), IL-10, IFNγ, and complement fragment 5a (C5a), 10 ng/mL each as indicated, and the percent increase in stimulated over baseline $CD11b_{Surface}$ as described in the Methods. Comparisons were made by ANOVA using the Holm-Sidak method for multiple comparisons; *** = P < 0.001.

reliability of the optimized test for detecting impaired GM-CSF signaling in heparinized human blood specimens. In healthy people, GM-CSF rapidly stimulated a robust translocation of pre-formed CD11b to the cell surface of neutrophils while in

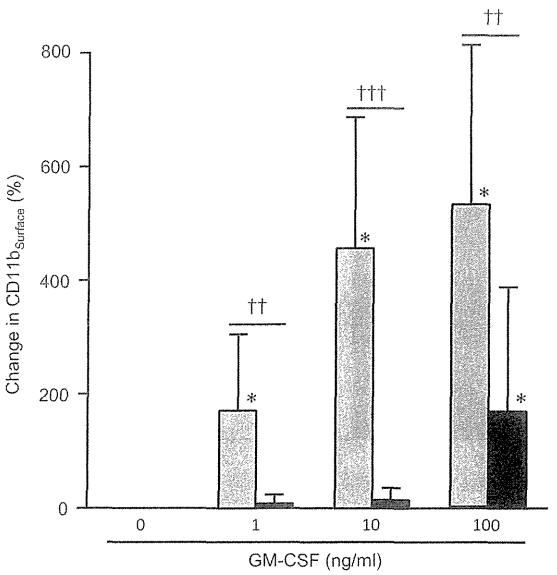


Fig. 8. Detection of impaired GM-CSF signaling in human blood specimens. Fresh, heparinized blood was collected from healthy people (n = 22; gray bars) or patients with autoimmune PAP (n = 5, closed bars) and percent increase of $CD11b_{Surface}$ over baseline as described in the Methods except that different GM-CSF concentrations were used for stimulation (indicated). Comparisons were made by the Mann-Whitney rank sum test with post-hoc analysis using Dunnett's test. comparison of corresponding values for healthy people or PAP patients to the un-stimulated control (no GM-CSF) are indicated; * = P < 0.05. Comparison of values for healthy people and PAP patients at each level of GM-CSF stimulation are also indicated; †† = P < 0.01, ††† = P < 0.001.

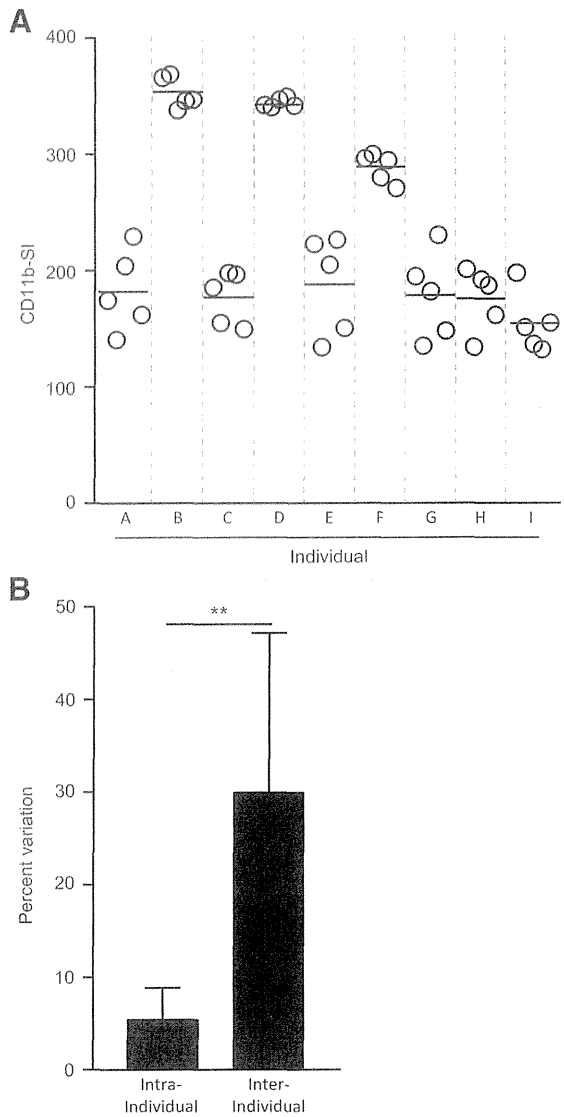


Fig. 9. Precision of the CD11b-SI test measurements in healthy people. A. Fresh, heparinized blood was collected from healthy people (n ≒ 9, A-I) and evaluated repeatedly in independent CD11b-SI test procedures (n = 5 per person). Shown are the individual determinations and mean for each individual (separated by gray dashed lines). Each symbol represents an independent determination. B. Evaluation of the percent variation among repeated determinations of CD11b-SI within the same subject (intra-subject) or between different subjects (inter-subject), calculated as described in the Methods. Comparison was made using Student's t test; ** = P < 0.01.

patients with autoimmune PAP, this translocation response was blocked. The assay performed very well in distinguishing impaired GM-CSF signaling in autoimmune PAP patients from normal signaling in healthy people for which the sensitivity and specificity were both 100%.

These results help establish a basis for the routine clinical use of blood testing for the differential diagnosis of PAP. The serum GM-CSF autoantibody ELISA test (GMAb ELISA) used to measure GM-CSF autoantibodies (Schoch et al., 2002) was recently optimized and found to have a sensitivity and specificity of 100% for diagnosis of autoimmune PAP (Uchida et al., 2014). The diagnostic use of the GMAb ELISA was

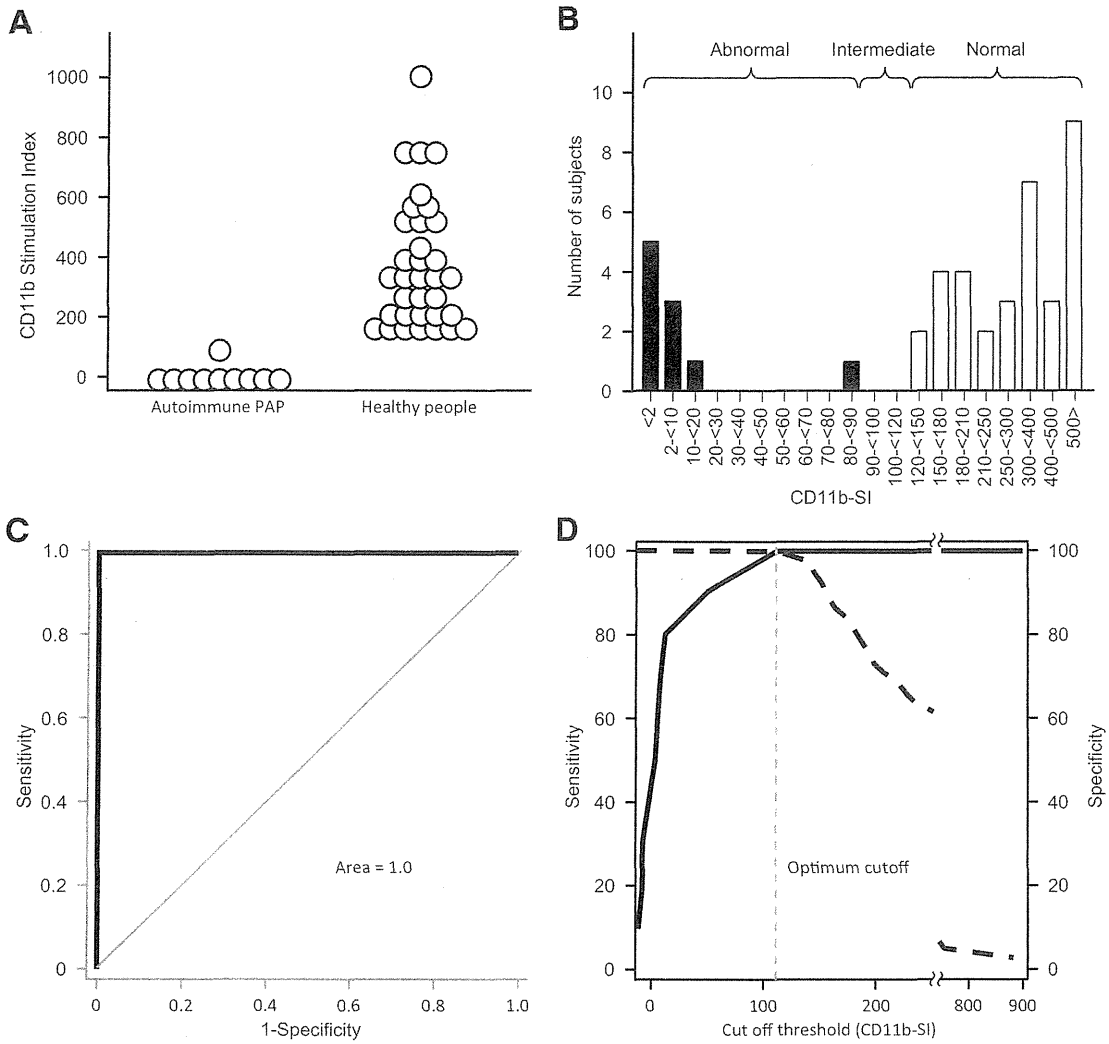


Fig. 10. Measurement and ROC Analysis of CD11b-SI in autoimmune PAP patients and healthy people. A. CD11b-SI test values in autoimmune PAP patients (10) and healthy people (34) measured as described in the Methods. B. Histogram of the frequency distribution of CD11b-SI test values in healthy people (open bars) and autoimmune PAP patients (filled bars). C-D. Receiver operating characteristic (ROC) curve analysis of CD11b-SI results for 10 autoimmune PAP patients and 34 healthy people. Standard ROC analysis was performed to determine the sensitivity and specificity for the data shown in Panel A. The are under the curve was 1.0 (C), and, at a cut off value for CD11b-SI of 112 determined by the software, the sensitivity and specificity of the CD11b-SI were both 100% (D).

supported by the demonstration that GM-CSF autoantibodies are the cause and not an associated epiphenomenon of autoimmune PAP (Sakagami et al., 2009, 2010). Support was also provided by a critical threshold of GM-CSF autoantibodies (Bendtzen et al., 2007) above which the risk of PAP is increased in non-human primates injected with patient-derived GM-CSF autoantibodies (Sakagami et al., 2010) and in humans (Uchida et al., 2009) and by ROC curve analysis confirming the value of the critical threshold (~5 μg/ml) using the optimized GMAb ELISA (Uchida et al., 2014). Notwithstanding, while the latter demonstrated most healthy people and autoimmune PAP patients have GMAb ELISA test results below 3 μg/ml or above 9 μg/ml, respectively, it identified an intermediate concentration range (>3, <9 μg/ml) in which diagnostic utility may be reduced by proximity to the critical threshold. The CD11b-SI test, by measuring impaired GM-CSF signaling in whole blood, can be used to determine the functional significance of an increased GM-CSF autoantibody level when the GMAb ELISA test result is near the critical threshold. Results identified a CD11b-SI of 112 as the optimal cutoff value for distinguishing specimens known to have impaired GM-CSF signaling (e.g., autoimmune PAP) from those of healthy people. However, as a conservative approach to test interpretation, we recommend that test results between 90 and 120 be 'read' as intermediate since data from healthy people or PAP patients to confirm this cutoff were not available in this range. Additional studies may be useful in further evaluating the accuracy, precision, ruggedness of the CD11b-SI for routine clinical use as a diagnostic test. In seven patients with hereditary PAP caused by disruption of GM-CSF receptor function, we found no significant response to GM-CSF stimulation using the CD11b-SI test (Suzuki et al., 2010). Thus, the combination of compatible radiographic findings, a normal GMAb ELISA test result and an abnormal CD11b-SI test result suggest a diagnosis of hereditary PAP. Further, since stimulation at very high concentrations of GM-CSF can stimulate an increase in

$CD11b_{Surface}$ in autoimmune PAP but likely not in hereditary PAP, the use of additional higher concentrations may be useful in the differential diagnosis of hereditary versus autoimmune PAP. However, further studies will be needed to explore and validate such an approach.

The CD11b-SI test may also be useful in other clinical or clinical research settings. For example, since $CD11b_{Surface}$ reflects neutrophil activation, it may be useful in patients with increased GM-CSF bioactivity such as serious infectious or inflammatory diseases (Shakoor and Hamblin, 1992; Palmer and Hamblin, 1993; Cook et al., 2013). Finally, it may be useful as an outcome measure in clinical trials aimed at reducing GM-CSF bioactivity, e.g., in autoimmune or inflammatory diseases (Cook et al., 2013), or the functional evaluation of cross-reacting anti-drug antibodies in individuals treated with recombinant human GM-CSF (Wadhwa et al., 1999). Finally, since this assay measures GM-CSF neutralizing capacity in the serum, it may be useful to monitor autoimmune PAP patients for spontaneous improvement or remission as expected if the GMAb were to fall below critical threshold (Uchida et al., 2009, 2014).

One limitation of the CD11b-SI test is the short time period after phlebotomy during which the test must be conducted in order to obtain useful results. The GM-CSF-stimulated increase in $CD11b_{Surface}$ begins to diminish by 1 hour after phlebotomy, and is markedly blunted by 6 hours, and near zero by 24 hours. This effect is due to an increase in baseline $CD11b_{Surface}$ caused by generation of lysophosphatidylcholines during blood storage, which have potent priming effect on neutrophils (Silliman et al., 1994). Neutrophils undergo apoptosis spontaneously with time after phlebotomy, however, this is not significant at times less than three hours (Homburg et al., 1995; Uchida et al., 2007). These time-dependent effects can be overcome by conducting the GM-CSF stimulation component of the test followed by immunostaining and blood fixation within 1 hour of phlebotomy, after which flow cytometric analysis can be delayed for up to 6 days without compromising the results. Another limitation is the sensitivity of $CD11b_{Surface}$ to changes in the temperature at which blood is kept before testing – i.e., placement on ice followed by warming to either room temp. or 37 °C, which has been reported previously (Shalekoff et al., 1998). In contrast, maintaining blood at 25 or 37 °C gave acceptable results similar to a previous report (Youssef et al., 1995). Use of EDTA as the anticoagulant also reduced the GM-CSF- stimulated increase in $CD11b_{Surface}$, consistent with the contribution of calcium ions to the translocation CD11b to the cell surface (Silliman et al., 2003). Variation in flow cytometer setting-dependent effects (e.g., variable laser output and machine settings) is a limitation (not evaluated here) that could significantly affect test results from day to day, especially if results are reported as a continuous variable corresponding to the numeric value of the CD11b-SI and interpreted based on normal and abnormal ranges of the test result. Finally, the magnitude of the GM-CSF-stimulated $CD11b_{Surface}$ (Fig. 8) and the ability to detect impaired GM-CSF signaling in blood specimens from autoimmune PAP patients (Bendtzen et al., 2007) varied with the concentration of GM-CSF used for stimulation. Thus, $CD11b_{Surface}$ measurement is highly sensitive to pre-analytical conditions of blood preparation and storage as noted in prior efforts to standardize the measurement of neutrophil $CD11b_{Surface}$ (Latger-Cannard et al., 2004).

Our approach to these limitations has been to standardize/control the CD11b-SI assay with respect to each variable. In our standardized CD11b-SI test, blood is collected into sodium heparin- containing phlebotomy tubes, kept at room temperature (~25 °C), stimulated with GM-CSF at a standard concentration (10 ng/ml) within 1 hour after phlebotomy, stained and immediately fixed. Blood from a healthy donor is collected and evaluated in parallel to control for day-to-day, machine, and inter-operator variability. Samples are then subjected to flow cytometric analysis as soon as possible usually in <24 hours, the data are expressed as a percent increase from baseline in $CD11b_{Surface}$ of the GM-CSF-stimulated sample. The test result is reported as a dichotomous variable - either "positive" or "negative" for GM-CSF signaling. These conditions have permitted the CD11b-SI test to be performed rapidly and reliably using whole blood specimens without any purification steps in the context of basic or clinical research. Because neutrophils in autoimmune PAP patients have normal ultrastructure, express expected phenotypic markers, and increase $CD11b_{Surface}$ after removal of GM-CSF autoantibodies, impaired CD11b-SI test results are interpreted as demonstrating the presence of functional GM-CSF autoantibodies (or the neutralizing capacity of whole blood) (Uchida et al., 2007, 2009). Consequently, we have used the CD11b-SI to evaluate impaired GM-CSF signaling in autoimmune PAP patients (Uchida et al., 2007, 2009), animal autoimmune PAP model with non-human primates induced by passive transfer of GM-CSF autoantibodies purified from patients (Sakagami et al., 2009, 2010), and other inflammatory diseases (Han et al., 2009).

Acknowledgements

We thank Carrie Stevens for help with collection of human blood specimens. This work was supported by grants from the National Heart Lung and Blood Institute (R01 HL085453; B.C.T.), National Center for Research Resources (U54 RR0198498, B.C.T. and K.U.), the National Institute for Health and Human Development, Japan Society for the Promotion of Science (A232490720001, B24390364, Y.Y. and K.U.), and Japan Ministry of Health Labor and Welfare (H24-Nanchitou(Nanchi)-Ippan-035, H24-Rinkensui-Ippan-003, K.U.)

References

Anonymous, 2001. Guidance for industry bioanalytical method validation. Guidence Documents, vol. 2012. U.S. Department of Health and Human Services, Food and Drug Administration, Center for Drug Evaluation and Research (CDER), Center for Veterinary Medicine (CVM), Bethesda.

Bendtzen, K., Svenson, M., Hansen, M.B., Busch, T., Bercker, S., Kaisers, U., Uchida, K., Beck, D.C., Trapnell, B.C., 2007. GM-CSF autoantibodies in pulmonary alveolar proteinosis. N. Engl. J. Med. 356, 2001.

Condliffe, A.M., Kitchen, E., Chilvers, E.R., 1998. Neutrophil priming: pathophysiological consequences and underlying mechanisms. Clin. Sci. (Lond.) 94, 461.

Cook, A.D., Pobjoy, J., Sarros, S., Steidl, S., Durr, M., Lacey, D.C., Hamilton, J.A., 2013. Granulocyte-macrophage colony-stimulating factor is a key mediator in inflammatory and arthritic pain. Ann. Rheum. Dis. 72, 265.

Dranoff, G., Crawford, A.D., Sadelain, M., Ream, B., Rashid, A., Bronson, R.T., Dickersin, G.R., Bachurski, C.J., Mark, E.L., Whitsett, J.A., et al., 1994. Involvement of granulocyte-macrophage colony-stimulating factor in pulmonary homeostasis. Science 264, 713.

Graves, V., Gabig, T., McCarthy, L., Strour, E.F., Leemhuis, T., English, D., 1992. Simultaneous mobilization of Mac-1 (CD11b/CD18) and formyl peptide chemoattractant receptors in human neutrophils. Blood 80, 776.

Hamilton, J.A., 2008. Colony-stimulating factors in inflammation and autoimmunity. Nat. Rev. Immunol. 8, 533.

Han, X., Uchida, K., Jurickova, I., Koch, D., Willson, T., Samson, C., Bonkowski, E., Trauernicht, A., Kim, M.O., Tomer, G., Dubinsky, M., Plevy, S., Kugathsan, S., Trapnell, B.C., Denson, L.A., 2009. Granulocyte-macrophage colony-stimulating factor autoantibodies in murine ileitis and progressive ileal Crohn's disease. Gastroenterology 136 (1261–71), e1.

Homburg, C.H., de Haas, M., von dem Borne, A.E., Verhoeven, A.J., Reutelingsperger, C.P., Roos, D., 1995. Human neutrophils lose their surface FcγRIII and acquire annexin V binding sites during apoptosis in vitro. Blood 85, 532.

Ishii, H., Trapnell, B.C., Tazawa, R., Inoue, Y., Akira, M., Kogure, Y., Tomii, K., Takada, T., Hojo, M., Ichiwata, T., Goto, H., Nakata, K., 2009. Comparative study of high-resolution CT findings between autoimmune and secondary pulmonary alveolar proteinosis. Chest 136, 1348.

Kitamura, T., Tanaka, N., Watanabe, J., Uchida, Kanegasaki, S., Yamada, Y., Nakata, K., 1999. Idiopathic pulmonary alveolar proteinosis as an autoimmune disease with neutralizing antibody against granulocyte/macrophage colony-stimulating factor. J. Exp. Med. 190, 875.

Lang, R.A., Metcalf, D., Cuthbertson, R.A., Lyons, I., Stanley, E., Kelso, A., Kannourakis, G., Williamson, D.J., Klintworth, G.K., Gonda, T.J., et al., 1987. Transgenic mice expressing a hemopoietic growth factor gene (GM-CSF) develop accumulations of macrophages, blindness, and a fatal syndrome of tissue damage. Cell 51, 675.

Latger-Cannard, V., Besson, I., Doco-Lecompte, T., Lecompte, T., 2004. A standardized procedure for quantitation of CD11b on polymorphonuclear neutrophil by flow cytometry: potential application in infectious diseases. Clin. Lab. Haematol. 26, 177.

Lieschke, G.J., Burgess, A.W., 1992. Granulocyte colony-stimulating factor and granulocyte-macrophage colony-stimulating factor (1). N. Engl. J. Med. 327, 28.

Martinez-Moczygemba, M., Doan, M.L., Elidemir, O., Fan, L.L., Cheung, S.W., Lei, J.T., Moore, J.P., Tavana, G., Lewis, L.R., Zhu, Y., Muzny, D.M., Gibbs, R.A., Huston, D.P., 2008. Pulmonary alveolar proteinosis caused by deletion of the GM-CSFRalpha gene in the X chromosome pseudoautosomal region 1. J. Exp. Med. 205, 2711.

Nogee, L.M., 2010. Genetic basis of children's interstitial lung disease. Pediatr. Allergy Immunol. Pulmonol. 23, 15.

Palmer, S., Hamblin, A.S., 1993. Increased CD11/CD18 expression on the peripheral blood leucocytes of patients with HIV disease: relationship to disease severity. Clin. Exp. Immunol. 93, 344.

Sakagami, T., Uchida, K., Suzuki, T., Carey, B.C., Wood, R.E., Wert, S.E., Whitsett, J.A., Trapnell, B.C., Luisetti, M., 2009. Human GM-CSF autoantibodies and reproduction of pulmonary alveolar proteinosis. N. Engl. J. Med. 361, 2679.

Sakagami, T., Beck, D., Uchida, K., Suzuki, T., Carey, B.C., Nakata, K., Keller, G., Wood, R.E., Wert, S.E., Ikegami, M., Whitsett, J.A., Luisetti, M., Davies, S., Krischer, J.P., Brody, A., Ryckman, F., Trapnell, B.C., 2010. Patient-derived granulocyte/macrophage colony-stimulating factor autoantibodies reproduce pulmonary alveolar proteinosis in nonhuman primates. Am. J. Respir. Crit. Care Med. 182, 49.

Schoch, O.D., Schanz, U., Koller, M., Nakata, K., Seymour, J.F., Russi, E.W., Boehler, A., 2002. BAL findings in a patient with pulmonary alveolar proteinosis successfully treated with GM-CSF. Thorax 57, 277.

Seymour, J.F., Doyle, I.R., Nakata, K., Presneill, J.J., Schoch, O.D., Hamano, E., Uchida, K., Fisher, R., Dunn, A.R., 2003. Relationship of anti-GM-CSF antibody concentration, surfactant protein A and B levels, and serum LDH to pulmonary parameters and response to GM-CSF therapy in patients with idiopathic alveolar proteinosis. Thorax 58, 252.

Shakoor, Z., Hamblin, A.S., 1992. Increased CD11/CD18 expression on peripheral blood leucocytes of patients with sarcoidosis. Clin. Exp. Immunol. 90, 99.

Shalekoff, S., Page-Shipp, L., Tiemessen, C.T., 1998. Effects of anticoagulants and temperature on expression of activation markers CD11b and HLA-DR on human leukocytes. Clin. Diagn. Lab. Immunol. 5, 695.

Silliman, C.C., Clay, K.L., Thurman, G.W., Johnson, C.A., Ambruso, D.R., 1994. Partial characterization of lipids that develop during the routine storage of blood and prime the neutrophil NADPH oxidase. J. Lab. Clin. Med. 124, 684.

Silliman, C.C., Elzi, D.J., Ambruso, D.R., Musters, R.J., Hamiel, C., Harbeck, R.J., Paterson, A.J., Bjornsen, A.J., Wyman, T.H., Kelher, M., England, K.M., McLaughlin-Malaxecheberria, N., Barnett, C.C., Aiboshi, J., Bannerjee, A., 2003. Lysophosphatidylcholines prime the NADPH oxidase and stimulate multiple neutrophil functions through changes in cytosolic calcium. J. Leukoc. Biol. 73, 511.

Suzuki, T., Sakagami, T., Rubin, B.K., Nogee, L.M., Wood, R.E., Zimmerman, S.L., Smolarek, T., Dishop, M.K., Wert, S.E., Whitsett, J.A., Grabowski, G., Carey, B.C., Stevens, C., van der Loo, J.C., Trapnell, B.C., 2008. Familial pulmonary alveolar proteinosis caused by mutations in CSF2RA. J. Exp. Med. 205, 2703.

Suzuki, T., Sakagami, T., Young, L.R., Carey, B.C., Wood, R.E., Luisetti, M., Wert, S.E., Rubin, B.K., Kevill, K., Chalk, C., Whitsett, J.A., Stevens, C., Nogee, L.M., Campo, I., Trapnell, B.C., 2010. Hereditary pulmonary alveolar proteinosis: pathogenesis, presentation, diagnosis, and therapy. Am. J. Respir. Crit. Care Med. 182, 1292.

Trapnell, B.C., Whitsett, J.A., 2002. Gm-CSF regulates pulmonary surfactant homeostasis and alveolar macrophage-mediated innate host defense. Annu. Rev. Physiol. 64, 775.

Trapnell, B.C., Whitsett, J.A., Nakata, K., 2003. Pulmonary alveolar proteinosis. N. Engl. J. Med. 349, 2527.

Uchida, K., Nakata, K., Trapnell, B.C., Terakawa, T., Hamano, E., Mikami, A., Matsushita, I., Seymour, J.F., Oh-Eda, M., Ishige, I., Eishi, Y., Kitamura, T., Yamada, Y., Hanaoka, K., Keicho, N., 2004. High-affinity autoantibodies specifically eliminate granulocyte-macrophage colony-stimulating factor activity in the lungs of patients with idiopathic pulmonary alveolar proteinosis. Blood 103, 1089.

Uchida, K., Beck, D.C., Yamamoto, T., Berclaz, P.Y., Abe, S., Staudt, M.K., Carey, B.C., Filippi, M.D., Wert, S.E., Denson, L.A., Puchalski, J.T., Hauck, D.M., Trapnell, B. C., 2007. GM-CSF autoantibodies and neutrophil dysfunction in pulmonary alveolar proteinosis. N. Engl. J. Med. 356, 567.

Uchida, K., Nakata, K., Suzuki, T., Luisetti, M., Watanabe, M., Koch, D.E., Stevens, C.A., Beck, D.C., Denson, L.A., Carey, B.C., Keicho, N., Krischer, J.P., Yamada, Y., Trapnell, B.C., 2009. Granulocyte/macrophage-colony-stimulating factor autoantibodies and myeloid cell immune functions in healthy subjects. Blood 113, 2547.

Uchida, K., Nakata, K., Carey, B., Chalk, C., Suzuki, T., Sakagami, T., Koch, D.E., Stevens, C., Inoue, Y., Yamada, Y., Trapnell, B.C., 2014. Standardized serum GM-CSF autoantibody testing for the routine clinical diagnosis of autoimmune pulmonary alveolar proteinosis. J. Immunol. Methods 402, 57.

Wadhwa, M., Skog, A.L., Bird, C., Ragnhammar, P., Lilljefors, M., Gaines-Das, R., Mellstedt, H., Thorpe, R., 1999. Immunogenicity of granulocyte-macrophage colony-stimulating factor (GM-CSF) products in patients undergoing combination therapy with GM-CSF. Clin. Cancer Res. 5, 1353.

Whitsett, J.A., Wert, S.E., Trapnell, B.C., 2004. Genetic disorders influencing lung formation and function at birth. Hum. Mol. Genet. 13, R207.

Youssef, P.P., Mantzioris, B.X., Roberts-Thomson, P.J., Ahern, M.J., Smith, M.D., 1995. Effects of ex vivo manipulation on the expression of cell adhesion molecules on neutrophils. J. Immunol. Methods 186, 217.