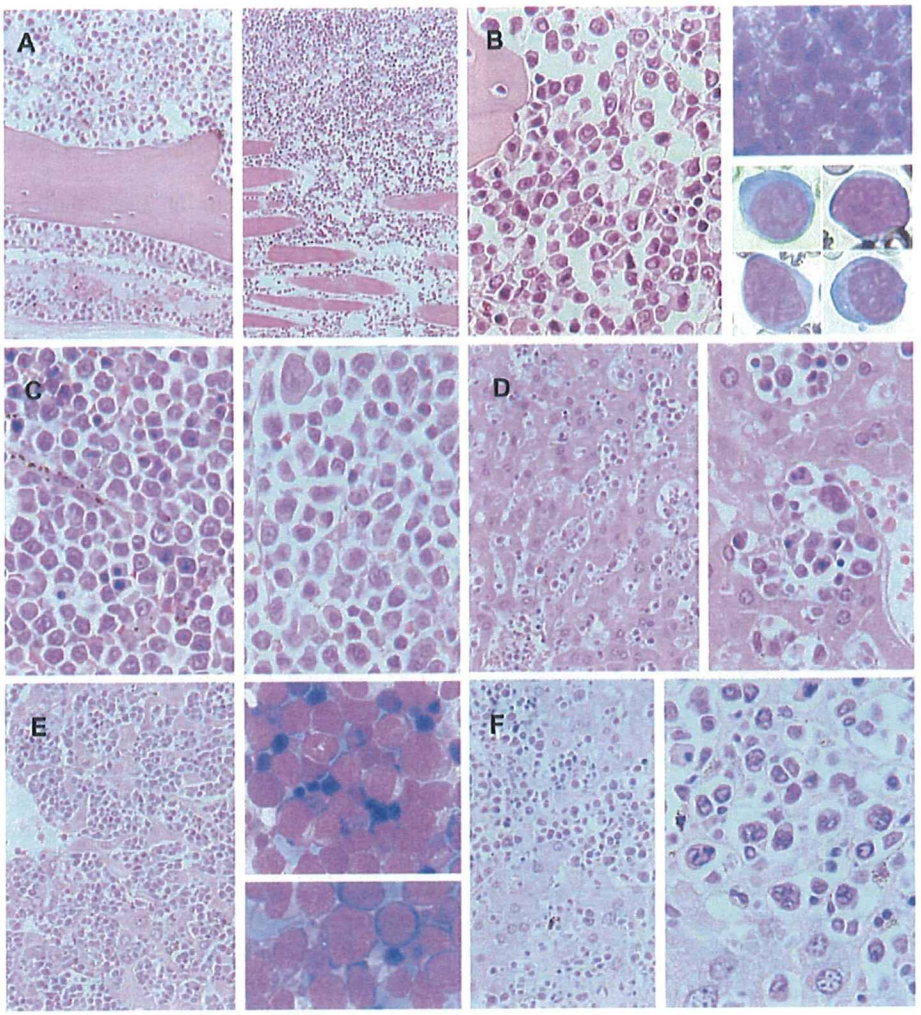

FIG. 7. Representative histopathological and cytopathological findings for AML developing after benzene exposure in heterozygous *Trp*53-deficient C3H/He and C57BL/6 mice: AML in femoral BM (A, left ×67) and its periosteal, intramuscular expansion, and infiltration into growth against surrounding soft part of femoral bone (A, right ×34). Higher magnification of atypical myeloid cells with widely heterogeneous size distribution and marked cellular atypia (B, left ×169). Imprint smear of hyperchromatic myeloblastic cells (B, inset top ×253) and representative characteristics of leukemic cells in smear showing atypical immature myeloblastic cells with trace evidence of intracytoplasmic peroxidase granulation (B, inset, upper row ×494 and lower row ×643). Atypical myeloid cellular component, proliferating in splenic white pulp for *Trp*53-deficient C3H/He mice (C, left ×169) and at higher magnification (C, right ×253). Hepatic trabecular infiltration of myeloid cells in liver of *Trp*53-deficient C3H/He mice (D, left ×67) and at higher magnification (D, right ×169). Atypical myeloid cell proliferation in liver of *Trp*53-deficient C57BL/6 mice (E, left ×67), atypical immature myeloid cells (E, right, top ×337), and tissue imprint smear from terminal stage of spleen with immature mononuclear myeloblastic cells (E, right, bottom ×337). Representative nonthymic malignant lymphoma, infiltrating into hepatic sinusoidal spaces (F, left ×67) with higher magnification of expansive growth of cerebriform bizarrely shaped cells (F, right ×169) in *Trp*53-deficient C57BL/6 mice.

low dose of benzene exposure, we found that heterozygous *Trp*53-deficient mice in both strains showed a higher than threshold incidence of HPNs at lower doses, as described in the "Results" section. We attribute this to the mechanism of *Trp*53-dependent repair for DNA damage induced by benzene exposure. Our second question related to the nonlinear plateau in the incidence of HPNs at high dose of benzene exposure. We found that *Trp*53-deficient mice in both strains produced a fairly high incidence of HPNs up to 100%, including 38% of AMLs in C3H/He mice exposed to benzene 300 ppm in comparison with an incidence of only 9% in wild-type mice exposed to the same dose. These results suggest that the nonlinear plateau in the incidence of HPNs at high benzene exposure may be caused by a decrease in neoplastic target cells due to *Trp*53-dependent escape from apoptosis in wild-type mice. In addition to benzene-mediated genotoxicity, the development of HPNs generally requires an epigenetic process that does not exhaust but maintains hematopoietic stem/progenitor cells, that is, the target cells for hematopoietic neoplastic development. An excessive decrease in the number

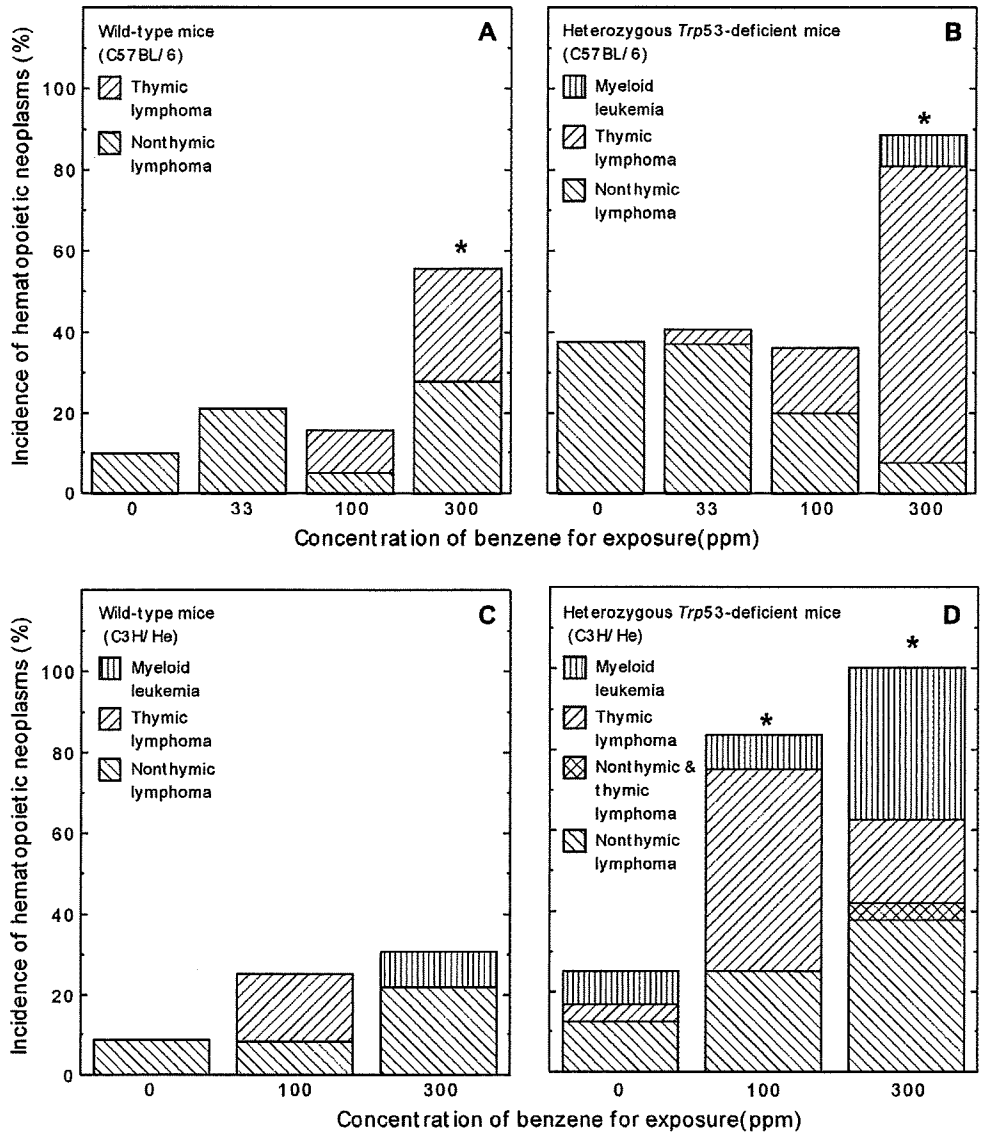

FIG. 8. Incidences of HPN histological types are shown in bar graphs. (A, B) C57BL/6 strain, wild-type mice (A) and heterozygous *Trp*53-deficient mice (B); (C, D) C3H/He strain, wild-type mice (C) and heterozygous *Trp*53-deficient mice (D). The incidence of HPNs is shown on the ordinate axis versus benzene exposure dose for the C57BL/6 strain (0, 33, 100, and 300 ppm) or the C3H/He strain (0, 100, and 300 ppm) on the horizontal axis of each graph. Histological types, such as AML, thymic lymphoma, nonthymic lymphoma associated with thymic lymphoma, and nonthymic lymphoma, are designated by inset legends in each figure. Incidences in heterozygous *Trp*53-deficient mice are higher than those in wild-type mice. Incidences in the 300-ppm exposure only (*) show statistically significant differences for both wild-type mice and heterozygous *Trp*53-deficient mice.

of hematopoietic stem/progenitor cells does not induce any hematopoietic neoplastic growth but rather induces irreversible aplastic anemia (Cronkite *et al.*, 1982). The Snyder-Cronkite benzene exposure protocol of 300 ppm, 6 h/day, 5 days/week, for the animal's lifetime or 16 weeks was originally aimed to not exhaust but maintain hematopoietic stem/progenitor cells. The exposure period was subsequently extended for the protocols up to 2 years in length (Huff *et al.*, 1989; NTP, 1986), but no substantial increase in the incidence of observed HPNs was reported. The exposure period applied in the present study was longer than in the original protocol by Cronkite *et al.* (1984, 1985, 1989) (16 weeks), which produced a higher incidence of HPNs owing to less exhaustion of hematopoietic stem/progenitor cells even in wild-type mice in both C57BL/6 and C3H/He strains. The relationship between the incidence of HPNs and the benzene exposure dose, however, showed a maximum increase to plateau with benzene exposure at less than 300 ppm (Figs. 2A and 2D). It, thus, appears that the

number of stem/progenitor cells available for targeting at 300 ppm in C3H/He mice is practically marginal not only for thymic lymphomas but also for all HPNs.

The potential for inducing HPNs seems to be limited in wild-type mice, as shown by the present protocol in both C57BL/6 and C3H/He strains as well as in reports by Huff *et al.* (1989) and the NTP (1986). However, we noted enhanced induction of HPNs after benzene exposure in *Trp*53-deficient mice and attributed this to arrest of the stem cell–specific cell cycle possibly owing to the genotoxicity induced by benzene exposure. Moreover, owing to *Trp*53 deficiency, benzene exposure in excess of 300 ppm appears to suppress the induction of HPNs as evidenced by the incidence of thymic lymphomas in heterozygous *Trp*53-deficient mice (Fig. 8D). A nonlinear limited increase and plateaued increase in the incidence of HPNs were also confirmed for the higher incidence of HPNs in *Trp*53-deficient mice with an impaired repair system. Regarding the known association between lower benzene toxicity and higher LD_{50} values, the results imply a trend based on the possible loss of progenitor cell–specific target cells for HPNs, that is, hematopoietic progenitor cells at higher benzene exposures (Yoon *et al.*, 2002).

*Trp*53-deficient mice develop undifferentiated immature HPNs (Yoshida *et al.*, 2002), which are attributed to the failure of *Trp*53 expression to regulate the differentiation process in myeloid cells (Feinstein *et al.*, 1992; Kastan *et al.*, 1991; Skorski *et al.*, 1996; Soddu *et al.*, 1994). As reported previously for radiation-induced AML in *Trp*53-deficient mice (Yoshida *et al.*, 2002, 2007), such AML tends to be characterized by a high incidence of stem cell leukemias and/or blastic leukemias, and there are traces of myeloid differentiation in homozygous *Trp*53-deficient mice with or without radiation exposure. Interestingly, the leukemia developing in *Trp*53-deficient mice after benzene exposure also showed less differentiation in the present study. Such reductions in differentiation are not seen in other thymic or nonthymic lymphomas. However, we were unable to confirm those findings here owing to insufficient data analysis of the precise level of differentiation since differentiation biomarkers for thymic and nonthymic lymphomas were not applied in the present study.

Third, the last issue is why benzene-induced HPNs are not leukemic, but largely thymic and nonthymic lymphomatous in mice (Cronkite *et al.*, 1985; Huff *et al.*, 1989), whereas most of the HPNs that develop after benzene exposure in humans are AMLs (Aksoy *et al.*, 1974; Delore and Borgomano, 1928; Vigliani and Forni, 1976). This query relating to the experimental development of leukemias in the narrow exposure dose range of benzene-induced HPNs has not been satisfactorily answered to date. In the present study, we found a marked difference between C57BL/6 and C3H/He mice in the incidence of different types of HPNs. Specifically, thymic lymphomas were predominantly induced in C57BL/6 mice, whereas nonthymic lymphomas were predominantly induced in C3H/He mice. Our findings may be supported by the gene expression differences reported for these strains after benzene exposure since the gene expression profiles in both strains were, to some extent, reciprocal for some cell cycle–regulating genes (data not shown). Comparable differences were also observed in the incidence of AMLs. Similar to findings following radiation exposure, C3H/He mice, which are prone to developing AMLs, tended to develop AMLs following benzene exposure.

An exposure-dependent limited increase was again observed in the incidence of AMLs up to 37.5% in *Trp*53-deficient C3H/He mice, and AMLs also developed even in wild-type C3H/He mice when exposed to 300 ppm. However, only two *Trp*53-deficient C57BL/6 mice developed AML at 300 ppm. This implies that there is a potential leukemogenicity not only in the C3H/He strain but also in the C57BL/6 strain, although in the C3H/He strain such leukemogenicity is associated more with an as-yet–undefined genetic background for induction of AMLs.

We noted a few C57BL/6 mice with myeloproliferative and/or myelodysplastic syndrome in the 33-ppm exposure group. This suggests that the protocol of 33-ppm exposure was insufficient for inducing HPNs since these syndromes are considered to be a preleukemic hematopoietic disorder.

FUNDING

Grants-in-Aid for Scientific Research C (15510064 and 18510066); the Ministry of Health, Labor and Welfare, Japan—Research Fund (H19-Chemistry 003); National Institute of Health Sciences.

ACKNOWLEDGMENTS

We thank Ms E. Tachihara, Mr K. Terasaka, Ms Y. Kondo, Ms C. Aoyagi, Ms Y. Usami, Ms Y. Shinzawa, and Ms M. Uchiyama for excellent technical assistance; Ms Y. Kikuchi, M. Yoshizawa, and Ms M. Hojo for secretarial assistance; and Ms Lee Seaman of Seaman Medical, Inc., for her lucid technical editing and advice.

REFERENCES

Aksoy, M., Erdem, S., and DinCol, G. (1974). Leukemia in shoe-workers exposed chronically to benzene. *Blood* **44,** 837–841.

Cabot, R. C. (1927). Case 13321 bleeding from the gums. *Boston M. & S.J.* **197,** 236.

Cronkite, E. P., Bullis, J., Inoue, T., and Drew, R. T. (1984). Benzene inhalation produces leukemia in mice. *Toxicol. Appl. Pharmacol.* **75,** 358–361.

Cronkite, E. P., Drew, R. T., Inoue, T., and Bullis, J. E. (1985). Benzene hematotoxicity and leukemogenesis. *Am. J. Ind. Med.* **7,** 447–456.

Cronkite, E. P., Drew, R. T., Inoue, T., Hirabayashi, Y., and Bullis, J. E. (1989). Hematotoxicity and carcinogenicity of inhaled benzene. *Environ. Health Perspect.* **82,** 97–108.

Cronkite, E. P., Inoue, T., Carsten, A. L., Miller, M. E., Bullis, J. E., and Drew, R. T. (1982). Effects of benzene inhalation on murine pluripotent stem cells. *J. Toxicol. Environ. Health* **9,** 411–421.

Delore, P., and Borgomano, C. (1928). Leucémie aiguë au cours de l'intoxication benzénique. Sur l'origine toxique de certaines leucémies aiguës et leurs relations avec les anémies graves. *J. de méd de Lyon* **9,** 227–233.

Feinstein, E., Gale, R. P., Reed, J., and Canaani, E. (1992). Expression of the normal p53 gene induces differentiation of K562 cells. *Oncogene* **7,** 1853–1857.

French, J. E., Lacks, G. D., Trempus, C., Dunnick, J. K., Foley, J., Mahler, J., Tice, R. R., and Tennant, R. W. (2001). Loss of heterozygosity frequency at the Trp53 locus in p53-deficient (+/-) mouse tumors is carcinogen- and tissue-dependent. *Carcinogenesis* **22,** 99–106.

Frith, C. H., Ward, J. M., Harleman, J. H., Stromberg, P. C., Halm, S., Inoue, T., and Wright, J. A. (2001). Hematopoietic system. In *International Classification of Rodent Tumors: The Mouse* (U. Mohr, Ed.), pp. 417–451. Springer-Verlag Berlin, Heidelberg, Berlin.

Haines, B. B., Ryu, C. J., Chang, S., Protopopov, A., Luch, A., Kang, Y. H., Draganov, D. D., Fragoso, M. F., Paik, S. G., Hong, H. J., *et al.* (2006). Block of T cell development in P53-deficient mice accelerates development of lymphomas with characteristic RAG-dependent cytogenetic alterations. *Cancer Cell* **9,** 109–120.

Harvey, M., McArthur, M. J., Montgomery, C. A., Jr., Butel, J. S., Bradley, A., and Donehower, L. A. (1993). Spontaneous and carcinogen-induced tumorigenesis in p53-deficient mice. *Nat. Genet.* **5,** 225–229.

Hirabayashi, Y., Inoue, T., Suda, Y., Aizawa, S., Ikawa, Y., and Kanisawa, M. (1992). Hemopoietic neoplasms in lethally irradiated mice repopulated with bone marrow cells carrying the human c-myc oncogene: A repopulation assay. *Exp. Hematol.* **20,** 167–172.

Hirabayashi, Y., Matsuda, M., Aizawa, S., Kodama, Y., Kanno, J., and Inoue, T. (2002). Serial transplantation of p53-deficient hemopoietic progenitor cells to assess their infinite growth potential. *Exp. Biol. Med. (Maywood)* **227,** 474–479.

Hirabayashi, Y., Yoshida, K., Aizawa, S., Kodama, Y., Kanno, J., Kurokawa, Y., Yoshimura, I., and Inoue, T. (2003). Evaluation of nonthreshold leukemogenic response to methyl nitrosourea in p53-deficient C3H/He mice. *Toxicol. Appl. Pharmacol.* **190,** 251–261.

Huff, J. E., Haseman, J. K., DeMarini, D. M., Eustis, S., Maronpot, R. R., Peters, A. C., Persing, R. L., Chrisp, C. E., and Jacobs, A. C. (1989). Multiple-site carcinogenicity of benzene in Fischer 344 rats and B6C3F1 mice. *Environ. Health Perspect.* **82,** 125–163.

Kastan, M. B., Radin, A. I., Kuerbitz, S. J., Onyekwere, O., Wolkow, C. A., Civin, C. I., Stone, K. D., Woo, T., Ravindranath, Y., and Craig, R. W. (1991). Levels of p53 protein increase with maturation in human hematopoietic cells. *Cancer Res.* **51,** 4279–4286.

Kemp, C. J., Wheldon, T., and Balmain, A. (1994). p53-deficient mice are extremely susceptible to radiation-induced tumorigenesis. *Nat. Genet.* **8,** 66–69.

Lan, Q., Zhang, L., Li, G., Vermeulen, R., Weinberg, R. S., Dosemeci, M., Rappaport, S. M., Shen, M., Alter, B. P., Wu, Y., *et al.* (2004). Hematotoxicity in workers exposed to low levels of benzene. *Science* **306,** 1774–1776.

Le Noir, M. M., and Claude, H. (1897). Sur un cas de purpura attribué a l'intoxication par la benzine. *Bull. Med. Soc. Hop. Paris* **14,** 1251–1260.

Li, G. X., Hirabayashi, Y., Yoon, B. I., Kawasaki, Y., Tsuboi, I., Kodama, Y., Kurokawa, Y., Yodoi, J., Kanno, J., and Inoue, T. (2006). Thioredoxin overexpression in mice, model of attenuation of oxidative stress, prevents benzene-induced hemato-lymphoid toxicity and thymic lymphoma. *Exp. Hematol.* **34,** 1687–1697.

MacDonald, J., French, J. E., Gerson, R. J., Goodman, J., Inoue, T., Jacobs, A., Kasper, P., Keller, D., Lavin, A., Long, G., *et al.* (2004). The utility of genetically modified mouse assays for identifying human carcinogens: A basic understanding and path forward. The Alternatives to Carcinogenicity Testing Committee ILSI HESI. *Toxicol. Sci.* **77,** 188–194.

Maltoni, C., Ciliberti, A., Cotti, G., Conti, B., and Belpoggi, F. (1989). Benzene, an experimental multipotential carcinogen: Results of the long-term bioassays performed at the Bologna Institute of Oncology. *Environ. Health Perspect.* **82,** 109–124.

Mouse Genome Informatics. (2009). *Trp53tm1Sia Targeted Allele Detail, ID: MGI: 1926340* Available at: http://www.informatics.jax.org/javawi2/servlet/WIFetch?page=alleleDetail&key=2871. Accessed June 4, 2009.

NTP. (1986). Toxicology and carcinogenesis studies of benzene (CAS No. 71-43-2) in F344/N rats and B6C3F1 mice (Gavage Studies). *Natl. Toxicol. Program Tech. Rep. Ser.* **289,** 1–277.

Penati, F., and Vigliani, E. C. (1938). Sul problema delle mielopatie aplastische pseudoaplastische e leucemiche d benzolo. *Ras. Med. Ind.* **9,** 345–361.

Santesson, C. G. (1897). Über chronischeVergiftungen mit Steinkohlenteer-benzin: vier Todesfälle. *Arch. Hyg. Berl.* **31,** 336–376.

Seki, M., Yoshida, K., Nishimura, M., and Nemoto, K. (1991). Radiation-induced myeloid leukemia in C3H/He mice and the effect of prednisolone acetate on leukemogenesis. *Radiat. Res.* **127,** 146–149.

Selling, L. (1910). A preliminary report on some cases of purpura hoemorrhagica due to benzol poisoning. *Bull. Johns Hopkins Hosp.* **21,** 33–37.

Skorski, T., Nieborowska-Skorska, M., Wlodarski, P., Perrotti, D., Martinez, R., Wasik, M. A., and Calabretta, B. (1996). Blastic transformation of p53-deficient bone marrow cells by p210bcr/abl tyrosine kinase. *Proc. Natl. Acad. Sci. U. S. A.* **93,** 13137–13142.

Smith, A. R. (1928). Chronic benzol poisoning among women industrial workers: A study of the women exposed to benzol fumes in six factories. *J. Indust. Hyg.* **10,** 73–93.

Snyder, C. A., Goldstein, B. D., Sellakumar, A. R., Bromberg, I., Laskin, S., and Albert, R. E. (1980). The inhalation toxicology of benzene: Incidence of hematopoietic neoplasms and hematotoxicity in ARK/J and C57BL/6J mice. *Toxicol. Appl. Pharmacol.* **54,** 323–331.

Snyder, R. (2002). Benzene and leukemia. *Crit. Rev. Toxicol.* **32,** 155–210.

Snyder, R. (2007). Benzene's toxicity: A consolidated short review of human and animal studies by HA Khan. *Hum. Exp. Toxicol.* **26,** 687–696.

Soddu, S., Blandino, G., Citro, G., Scardigli, R., Piaggio, G., Ferber, A., Calabretta, B., and Sacchi, A. (1994). Wild-type p53 gene expression induces granulocytic differentiation of HL-60 cells. *Blood* **83,** 2230–2237.

Storer, R. D., French, J. E., Haseman, J., Hajian, G., LeGrand, E. K., Long, G. G., Mixson, L. A., Ochoa, R., Sagartz, J. E., and Soper, K. A. (2001). P53+/- hemizygous knockout mouse: Overview of available data. *Toxicol. Pathol.* **29**(Suppl), 30–50.

Tsukada, T., Tomooka, Y., Takai, S., Ueda, Y., Nishikawa, S., Yagi, T., Tokunaga, T., Takeda, N., Suda, Y., Abe, S., *et al.* (1993). Enhanced proliferative potential in culture of cells from p53-deficient mice. *Oncogene* **8,** 3313–3322.

Vigliani, E. C., and Forni, A. (1976). Benzene and leukemia. *Environ. Res.* **11,** 122–127.

WHO. (2003). *Benzene in Drinking-Water. Background Document for Development of WHO Guidelines for Drinking-Water Quality.* WHO/SDE/WSH/03.04/24. Accessed June 4, 2009. Originally published in *Guidelines for drinking-water quality, 2nd ed. Vol. 2. Health criteria and other supporting information.* World Health Organization, Geneva.

Yagi, T., Tokunaga, T., Furuta, Y., Nada, S., Yoshida, M., Tsukada, T., Saga, Y., Takeda, N., Ikawa, Y., and Aizawa, S. (1993). A novel ES cell line, TT2, with high germline-differentiating potency. *Anal. Biochem.* **214,** 70–76.

Yoon, B. I., Hirabayashi, Y., Kawasaki, Y., Kodama, Y., Kaneko, T., Kanno, J., Kim, D. Y., Fujii-Kuriyama, Y., and Inoue, T. (2002). Aryl hydrocarbon receptor mediates benzene-induced hematotoxicity. *Toxicol. Sci.* **70,** 150–156.

Yoon, B. I., Hirabayashi, Y., Kawasaki, Y., Kodama, Y., Kaneko, T., Kim, D. Y., and Inoue, T. (2001). Mechanism of action of benzene toxicity: Cell cycle suppression in hemopoietic progenitor cells (CFU-GM). *Exp. Hematol.* **29,** 278–285.

Yoon, B. I., Li, G. X., Kitada, K., Kawasaki, Y., Igarashi, K., Kodama, Y., Inoue, T., Kobayashi, K., Kanno, J., Kim, D. Y., *et al.* (2003). Mechanisms of benzene-induced hematotoxicity and leukemogenicity: cDNA microarray analyses using mouse bone marrow tissue. *Environ. Health Perspect.* **111,** 1411–1420.

Yoshida, K., Aizawa, S., Watanabe, K., Hirabayashi, Y., and Inoue, T. (2002). Stem-cell leukemia: p53 deficiency mediated suppression of leukemic differentiation in C3H/He myeloid leukemia. *Leuk. Res.* **26,** 1085–1092.

Yoshida, K., Hirabayashi, Y., Wada, S., Watanabe, F., Watanabe, K., Aizawa, S., and Inoue, T. (2007). p53 (TRP53) deficiency-mediated antiapoptosis escape after 5 Gy X irradiation still induces stem cell leukemia in C3H/He mice: Comparison between whole-body assay and bone marrow transplantation (BMT) assay. *Radiat. Res.* **167,** 703–710.

Yoshida, K., Nojima, K., Seki, M., and Inoue, T. (1996). Radiation-induced myeloid leukemia following splenectomy in C3H/He mice. *J. Radiat. Res.* **37,** 380.

BMC Infectious Diseases

BioMed Central

Research article

Open Access

Quality assessment of an interferon-gamma release assay for tuberculosis infection in a resource-limited setting

Nguyen TL Hang[1], Naoki Ishizuka[2], Naoto Keicho*[2], Le T Hong[3], Do B Tam[3], Vu TX Thu[3], Ikumi Matsushita[2], Nobuyuki Harada[4], Kazue Higuchi[4], Shinsaku Sakurada[2] and Luu T Lien[3]

Address: [1]IMCJ-BMH Medical Collaboration Center, Bach Mai Hospital, Hanoi, Viet Nam, [2]International Medical Center of Japan, Research Institute, Tokyo, Japan, [3]Hanoi Tuberculosis and Lung Disease Hospital, Hanoi, Viet Nam and [4]Research Institute of Tuberculosis, Japan Anti-Tuberculosis Association, Tokyo, Japan

Email: Nguyen TL Hang - lehang0310@gmail.com; Naoki Ishizuka - naishi@ri.imcj.go.jp; Naoto Keicho* - nkeicho-tky@umin.ac.jp; Le T Hong - lethihong.hth@gmail.com; Do B Tam - dobangtam.hth@gmail.com; Vu TX Thu - vuthixuanthu.hth@gmail.com; Ikumi Matsushita - ikumi-tky@umin.ac.jp; Nobuyuki Harada - harada@jata.or.jp; Kazue Higuchi - higuchi@jata.or.jp; Shinsaku Sakurada - ssakura@mti.biglobe.ne.jp; Luu T Lien - luuthilien.hth@gmail.com

* Corresponding author

Published: 18 May 2009

BMC Infectious Diseases 2009, 9:66 doi:10.1186/1471-2334-9-66

Received: 20 February 2009
Accepted: 18 May 2009

This article is available from: http://www.biomedcentral.com/1471-2334/9/66

Abstract

Background: When a test for diagnosis of infectious diseases is introduced in a resource-limited setting, monitoring quality is a major concern. An optimized design of experiment and statistical models are required for this assessment.

Methods: Interferon-gamma release assay to detect tuberculosis (TB) infection from whole blood was tested in Hanoi, Viet Nam. Balanced incomplete block design (BIBD) was planned and fixed-effect models with heterogeneous error variance were used for analysis. In the first trial, the whole blood from 12 donors was incubated with nil, TB-specific antigens or mitogen. In 72 measurements, two laboratory members exchanged their roles in harvesting plasma and testing for interferon-gamma release using enzyme linked immunosorbent assay (ELISA) technique. After intervention including checkup of all steps and standard operation procedures, the second trial was implemented in a similar manner.

Results: The lack of precision in the first trial was clearly demonstrated. Large within-individual error was significantly affected by both harvester and ELISA operator, indicating that both of the steps had problems. After the intervention, overall within-individual error was significantly reduced ($P < 0.0001$) and error variance was no longer affected by laboratory personnel in charge, indicating that a marked improvement could be objectively observed.

Conclusion: BIBD and analysis of fixed-effect models with heterogeneous variance are suitable and useful for objective and individualized assessment of proficiency in a multistep diagnostic test for infectious diseases in a resource-constrained laboratory. The action plan based on our findings would be worth considering when monitoring for internal quality control is difficult on site.

Background

Assuring quality is essential for clinical laboratories in the field of infectious diseases. Beneficiaries are not only patients obtaining a diagnosis on site but also future patients receiving benefits of clinical research supported by qualified laboratories. Quality assurance in modern laboratories is realized by total quality management including external quality assurance (EQA) and internal quality control (IQC) [1-3].

In most resource-constrained countries, however, regulations on quality assurance have not been laid down by the authorities and accuracy and precision of clinical measurements have not been monitored systematically [4]. Under such disadvantageous circumstances, when important but rather complicated testing for infectious diseases is undertaken, we cannot easily be confident that the skill has been transferred and maintained properly until the procedure becomes familiar and stably performed in accordance with a desirable quality control system [5]. During this vulnerable period, how to assess proficiency of the testing effectively and objectively, and how to assure and improve the quality are open issues to be addressed.

Currently, immunoassay is commonly used to make a serological diagnosis of infectious diseases involving human immunodeficiency virus, a variety of hepatitis virus and other sexually transmitted or blood-borne pathogens [6,7], which are serious problems in the developing world. Enzyme linked immunosorbent assay (ELISA) is often used to make diagnosis of these diseases in the clinical laboratories. Because of the complexity of the method, however, quality control of these assay systems is challenging [8]. In this context, trend of point of care (POC) tests that facilitate immediate and on-site diagnosis as well as early treatment of infectious diseases has been emphasized [7]. However, their usage in resource-constrained countries is still hampered by high cost and difficulties in testing for high throughput screening and thus laboratory-based immunoassays would be irreplaceable in many fields.

Recently, a two-step immunoassay to detect tuberculosis (TB) infection has also been developed and used extensively [9]. It consists of whole blood stimulation with TB-specific antigens followed by measurement of interferon-gamma using ELISA. Our objective in the present study is to demonstrate that the quality of laboratory tests can be assessed objectively even in a resource-constrained laboratory if the optimum design of experiments and appropriate statistical models are chosen. As a result of this attempt, we experienced marked improvement of the quality of this multi-step immunoassay made by more than one laboratory staff member in a hospital of Viet Nam. We proposed a general plan to evaluate skills of laboratory staff members efficiently and quantitatively to perform qualified immuno-diagnostic testing especially for infectious diseases until such time as they establish a total quality management system by themselves.

Methods

Interferon-gamma release assay (IGRA) for diagnosis of TB infection

IGRA is a general method to measure interferon-gamma induced by *Mycobacterium tuberculosis*-specific antigens (TB-Antigen) for detecting TB infection. In the ELISA-based IGRA (QuantiFERON-TB Gold In-Tube™, Cellestis, Victoria, Australia), one milliliter of the whole blood was collected into the Nil tube for negative control, Mitogen for positive control, and TB-Antigen separately. The blood in the tubes was mixed and placed in the incubator for 18 hours at 37°C (Cool incubator NC-25B, Funakoshi, Tokyo, Japan). Approximately 200 μl of plasma were harvested from each tube after centrifugation (Kubota 2010, Kubota, Tokyo, Japan).

Interferon-gamma concentrations in the plasma were measured by ELISA, using microtiter plate washer and reader (Wellwash Plus Microplate Washer and Multiscan JX Microplate Reader, Thermo Electron Corporation, Vantaa, Finland) with the analysis software provided by the manufacturer (QuantiFERON-TB Gold Analysis Software, ver. 2.50, Cellestis). In this study, interferon-gamma concentrations obtained from this calculation were directly used for further analysis.

Study setting

Two trials were carried out in Hanoi TB and Lung Disease Hospital, Viet Nam. Between the first and second trial, statistical analysis was made and an intervention was planned to ensure counterchecking and correct questionable manipulations. Each trial consisted of two runs. In each run, three milliliters of blood were collected from volunteers after informed consent had been obtained. Study protocols using IGRA were approved by ethical committees of the Ministry of Health, Viet Nam and International Medical Center of Japan respectively.

Two laboratory members, A and B, performed either plasma harvest or ELISA operation or both: Harvest included labeling and placing plasma storage tubes properly and transferring plasma from centrifuged blood collection tubes to these tubes by pipetting. ELISA was a process including preparing reagents and transferring plasma samples into the microtiter plate. ELISA ended with calculation of interferon-gamma concentration. Because their roles were changed occasionally due to the limited manpower of the laboratory, their performance in both Harvest and ELISA was the subject to be analyzed.

Balanced incomplete block design (BIBD)

A single specimen obtained from routine blood collection was not sufficient to assess two staff members' performance. Because additional blood sampling was not easily accepted in many countries including Viet Nam, BIBD was attempted to obtain analytical information from small volume of plasma samples in this study: Of four possible combinations of harvester and ELISA operator, two combinations were cyclically chosen, using the limited amount of specimen. Allocation of observed combinations by BIBD in this study was described in Table 1. In each trial, there were two levels of Harvest (two different Harvesters), two levels of ELISA (two different ELISA operators) and 12 levels of Specimen (12 different blood donors).

Outliers

To identify outliers, Mahalanobis distance D was calculated, which took the distance from the mean and correlation into account [10]. When D > 2.0, the value of that observed pair was regarded as outlier.

A fixed-effect model and three-way analysis of variance (ANOVA)

To assess effects of factors of interest and error variance, we used a fixed-effect model:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk}$$

of which,

y_{ijk}: Interferon-gamma concentration in the plasma

μ: Grand mean of all measurements

α_i: Harvest with i levels: i = 1, 2 (= A and B)

β_j: ELISA with j levels: j = 1, 2 (= A and B)

γ_k: Blood specimen with k levels: k = 1, 2,..., 12

ε_{ijk}: Within-individual error; following normal distribution with mean = 0 and variance = σ^2: N (0, σ^2)

In this clinical setting, effects of interaction terms were not considered in the above model, because harvesting plasma and performing ELISA are independent steps and it is unlikely that the exchanging of staff roles in itself could increase the chances of error.

Analysis of heterogeneous error variance affected by a given factor

To determine whether individuals of Harvest or ELISA affect within-individual error, we assessed a fixed-effect model with heterogeneous variance of error in the following way:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ij}$$

where error follows the normal distribution $N(0, \sigma_{ij}^2)$.

Error variance affected by Harvesters was evaluated in the following formula:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{i.}$$

Similarly, the following formula was used for error variance affected by ELISA operators:

$$y_{ijk} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{.j}$$

Coefficient of variation (CV) before and after intervention

Error variance ε_{ijk} that included sources of Harvest and ELISA was calculated in a simple one-way ANOVA model adjusted by specimen. Based on the following formula, CVs of the two trials were assessed:

$$y = \mu + \gamma_k + \varepsilon_{ijk}$$

Table 1: Allocation of observed combinations of Harvester and ELISA operator.

Sample	Specimen*	Harvest	ELISA	Data	Sample	Specimen	Harvest	ELISA	Data
1	1	A	A	Observed	7	4	A	A	Observed
2	1	A	B	Observed		4	A	B	Not observed
	1	B	A	Not observed	8	4	B	A	Observed
	1	B	B	Not observed		4	B	B	Not observed
	2	A	A	Not observed		5	A	A	Not observed
	2	A	B	Not observed	9	5	A	B	Observed
3	2	B	A	Observed		5	B	A	Not observed
4	2	B	B	Observed	10	5	B	B	Observed
5	3	A	A	Observed		6	A	A	Not observed
	3	A	B	Not observed	11	6	A	B	Observed
	3	B	A	Not observed	12	6	B	A	Observed
6	3	B	B	Observed		6	B	B	Not observed

*To each specimen, two measurements were assigned. This layout was repeated twice by using different sets of specimens in each trial.

$$CV(\%) = \frac{\text{Root mean square error}}{\text{Mean}} \times 100$$

CV should not be larger than 20% in any types of immunoassay [8].

Assessment of heterogeneous variance between the two trials

To analyze overall within-individual error between the two trials statistically, we used a fixed-effect model with heterogeneous variance of error, under the assumption that α and β were fixed throughout the trials. The effect of each blood specimen γ was expected to be different between the two trials.

$$y_{ijk1} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk1} \text{(the first trial)}$$
$$\| \quad \| \quad \| \quad \|$$
$$y_{ijk2} = \mu + \alpha_i + \beta_j + \gamma_k + \varepsilon_{ijk2} \text{(the second trial)}$$

of which, ε_{ijk1} and ε_{ijk2} were within-individual errors of the first and the second trial respectively. On the above assumption, ε_{ijk1} and ε_{ijk2} would be heterogeneous error between the trials.

Calculation of Mahalanobis distance, three-way ANOVA and estimation of heterogeneous variance were performed by SAS version 9.1 (SAS Institute Cary, NC, USA). Differences in error variance of two trials and error variance affected by a given factor were considered to be significant when *P*-value was less than 0.05.

Results

Evaluation of outliers and three-way ANOVA in the first trial

Out of 72 measurements obtained from the first trial, seven outliers were identified: One was in Nil condition, three in TB-Antigen and three in Mitogen (Mahalanobis D = 2.64 to 4.69).

To assess effects of individuals for Harvest and ELISA and character of errors involved in the first trial, we first performed three-way ANOVA using a fixed-effect model, in which three factors, Harvest, ELISA and individual blood specimens may have possible effects on the interferon-gamma concentration respectively. This model decomposes the total variance into between-individual error (or bias) and within-individual error (or imprecision). Herein, "between-individual error" indicates deviation in interferon-gamma values caused by the difference between Harvesters or ELISA operators, and "within-individual error" represents fluctuation of interferon-gamma values measured by a single Harvester or ELISA operator.

As shown in Table 2, mean square error indicating magnitude of within-individual error was large in all conditions of the first trial, which was indicated by remarkably large CV (> 20%) for Nil, TB-Antigen and Mitogen. Furthermore, in the condition of Mitogen, the mean-square value directing the effect of ELISA, or "between-individual error", was significantly large ($P = 0.017$). In the other two conditions, the effects of ELISA and Harvest were also considerably large but did not reach significant levels, as compared with the corresponding mean square errors. These findings indicate that their performance is unstable. Problems specific to ELISA and Harvest should be considered, although not statistically significant in all conditions.

Analysis of heterogeneous error variance in the first trial

We then analyzed which factor affected within-individual error. Because two laboratory members were involved in each step of this experiment, we assumed that within-individual error, i.e. error variance, could be different depending on the personnel in each step. Thus, we chose a fixed-effect model with heterogeneous variance of error affected by Harvest and ELISA (Table 3).

In Nil condition, difference in error variance was statistically significant between Harvesters A and B ($P = 0.0040$), when error variance caused by ELISA operator was not considered. Difference of error variance caused by ELISA operators A and B was also significant ($P = 0.024$), when error variance caused by Harvester was not taken into account. These findings imply that under the model, the error variance was affected significantly by different Harvesters or ELISA operators, respectively.

Intervention

By means of the above-mentioned statistical analysis of the first trial, we identified several points to be improved: a) there was a considerable number of outliers. Within-individual error was large and between-individual error

Table 2: Three-way analysis of variance in the first trial.

	Nil	TB-Antigen	Mitogen
Mean (IU/ml)	0.7821	5.4013	15.3638
Harvest			
Mean Square	0.0000	1.5252	51.2656
F value	0.0000	0.2200	2.5600
P value	0.9984	0.6482	0.1404
ELISA			
Mean Square	1.8838	12.3026	161.3535
F value	1.2600	1.7800	8.0700
P value	0.2847	0.2112	0.0175
Error			
Mean Square	1.4741	6.8935	19.9916
Root Mean Square	1.2142	2.6255	4.4712
Coefficient of Variation (%)	155.2476	48.6099	29.1022

Table 3: Error variance affected by Harvester (left) and error variance affected by ELISA operator (right) in the first trial.

$\varepsilon i.$	Harvester	*P* value	$\varepsilon .j$	ELISA operator	*P* value
Nil	A:1.9150 B:0.0160	0.0040	Nil	A:0.0036 B:3.2723	0.0244
TB-Antigen	A:2.9897 B:9.7114	0.2546	TB-Antigen	A: 0.1270 B:15.2216	0.0830
Mitogen	A:33.5782 B: 5.6792	0.2780	Mitogen	A:41.1154 B: 3.0221	0.3584

was also comparably large, and b) within-individual error was affected by both Harvesters and ELISA operators at least when Nil was measured.

Based on these results, an intervention was introduced: 1) reviewing all procedures of Harvest and ELISA, 2) reconsidering and strengthening standard operation procedures, 3) checking working condition of machines, and 4) developing a checklist for countercheck. First, we attempted to find out which procedure of harvesting and ELISA operation would be unstable and all questionable manipulations were listed up. Essential laboratory skills, such as mixing the solution by pipetting, were reviewed. Secondly, standard operation procedures were rechecked and corrected seeing that the laboratory personnel were handling three blood collection tubes and three other plasma storage tubes from each blood donor at a time, they should take every care to identify the tubes during Harvest and ELISA and to confirm the right position of corresponding tubes. Thorough instruction for handling ELISA plates and tubes with manipulation of the pipette was given to avoid carry-over error or contamination. After intensive discussions, more attention was paid to basic laboratory practice and reduction of preventable mistakes. Thirdly, performance of the ELISA plate washer and reader and the quality of distilled water were also checked. Technical requirements from the manufacturer, such as temperature for reagent reservation, time of incubation, were strictly followed. Finally, a checklist for the countercheck of each step was developed for practical use.

General assessment by CV before and after intervention

To assess the overall improvement after intervention, CV was compared between the two trials. Because variation due to Harvest and ELISA was of interest, CV adjusted by the effect of specimens was calculated and used. The CV had decreased remarkably in each condition of the second trial, as compared with that of the first trial, indicating the overall improvement of test performance after intervention (Table 4).

Evaluation of outliers and three-way ANOVA in the second trial

In the second trial, only one outlier was seen in Nil condition (Mahalanobis D = 2.59); the number of outliers was lower than that of the first trial.

We then proceeded to analyze the change of parameters that had possibly contributed to overall improvement of test performance. As shown in Table 5, both mean square error and mean-square values showing effects of Harvest and of ELISA were markedly lower in the second trial. The former implies the decrease in within-individual error and the latter shows the reduction of between-individual error. The latter change was also clearly shown when differences of least square means between Harvesters and between ELISA operators in each condition of the second trial were compared with those in the first trial (Figure 1).

Analysis of heterogeneous error variance affected by harvester and ELISA operator in the second trial

In contrast to the first trial, there were no significant differences of error variance affected by Harvesters or ELISA operators (Table 6). This finding showed that the heterogeneous error variance indicating personnel-dependent unstableness was small enough in each step of the second trial.

Assessment of heterogeneous variance between the two trials

We further evaluated the decrease in overall within-individual error statistically. For this purpose, we used a fixed-effect model with heterogeneous variance between the two trials. Under the assumption that influence of Harvest and ELISA was not changed between the two trials, estimated overall error variances of the two trials were com-

Table 4: CV adjusted by specimen in the two trials.

	CV (%)	
Condition	1st trial	2nd trial
Nil	150.5036	2.1661
TB-Antigen	48.6219	2.3967
Mitogen	38.1630	9.8776

Table 5: Three-way analysis of variance in the second trial.

	Nil	TB-Antigen	Mitogen
Mean (IU/ml)	0.2308	0.3071	11.0017
Harvest			
Mean Square	0.0000	0.0000	3.4225
F value	1.0000	0.1000	3.2100
P value	0.3409	0.7572	0.1036
ELISA			
Mean Square	0.0000	0.0000	0.0770
F value	1.0000	0.4000	0.0700
P value	0.3409	0.5393	0.7937
Error			
Mean Square	0.0000	0.0001	1.1707
Root Mean Square	0.0050	0.0079	1.0330
Coefficient of Variation (%)	2.1661	2.5615	9.3898

pared. As shown in Table 7, values indicating the overall within-individual error in all conditions had significantly decreased in the second trial ($P < 0.0001$).

Discussion

We have demonstrated that a study design BIBD and statistical analysis using fixed-effect models with heterogeneous variance of error are useful for objective and quantitative assessment of laboratory testing for the first time. A series of experiments in our study clearly showed that proficiency of the personnel was improved by an appropriate intervention between the first and second trials of a two-step ELISA-based immunoassay for tuberculosis newly introduced to a resource-constrained laboratory.

Design of clinical experiments including block designs can be used to estimate effect of factors and their possible interaction [10]. In block designs including BIBD, introduction of blocks usually provides extra precision for comparison of other factors, while difference between blocks is of no intrinsic interest [10]. In our proficiency testing, variation of individual specimens was not the point of interest, but analysis of the other two factors, Harvest and ELISA was of importance. Roles of laboratory members are occasionally changed because of limited manpower. In such a case, our analysis is indispensable for assessment of their individual skills in each step of the testing, since this kind of approach has not been evaluated by the conventional IQC methods [11].

Previous studies in clinical fields other than laboratory medicine showed the advantage of BIBD over the sample size [12-14]. In the present study, this design enabled us to evaluate essential components of the blood testing procedure systematically without collecting an extra specimen from each donor. If all combinations of Harvesters and ELISA operators were to be tested at the same time, a twice-larger volume of blood should be collected from each volunteer, however, obtaining consent of this often causes difficulties in a country where blood sampling is not easily accepted. We have shown furthermore that this design is suitable for clinical settings in which many different specimens are to be handled at the same time.

In the first trial before intervention, we found that within-individual error was large and between-individual error tended to be so. However, a number of outliers also affected both within- and between-individual errors. The cause of outliers was probably due to mixing up of speci-

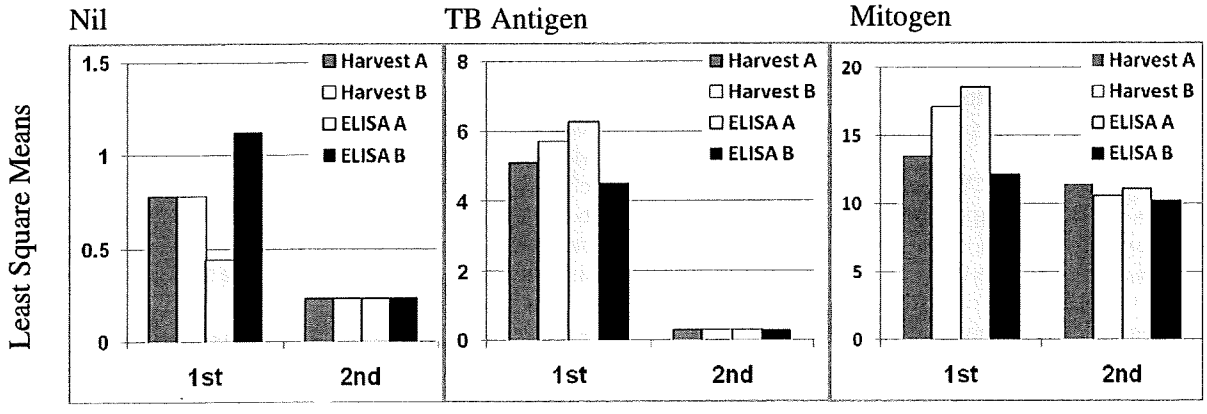

Figure 1
Least square means of measurements in the first and the second trials. Differences in least square means between Harvesters and between ELISA operators in the conditions of Nil, TB-Antigen and Mitogen in the second trial were compared with those in the first trial.

Table 6: Error variance affected by Harvester (left) and error variance affected by ELISA operator (right) in the second trial.

$\varepsilon i.$	Harvester	*P* value	$\varepsilon .j$	ELISA operator	*P* value
Nil	NE* (A > B)		Nil	A:0.000014 B:0.000035	0.4832
TB-Antigen	A:0.0001 B:0.00002	0.2391	TB-Antigen	NE (A > B)	
Mitogen	A:2.1184 B: 0.1788	0.3291	Mitogen	A:1.9065 B: 0.2747	0.33347

*NE = not estimable by this calculation.

men tubes or contamination of samples resulting from unfamiliar handling of multiple samples, although this was not easily determined [15,16]. Using a fixed-effect model with the heterogeneity of error variance, we further illustrated that within-individual error was affected by Harvesters and ELISA operators. The results indicated that there were problems with both steps of Harvest and ELISA, and with both laboratory members, and this represented a strong motivation to improve the skills of the laboratory personnel in both steps of Harvest and ELISA.

After timely intervention including checkup of all steps and standard operation procedures, marked improvement was observed in all parameters including CV, a general parameter for precision of measurements [8]. In case of IGRA in this study, CV should be kept less than 10% [17,18] and in the second trial, this criterion was met satisfactorily.

We propose as a consequence the following action plan to improve diagnostic capacity in resource-constrained settings. This could be generalized not only for complicated immunoassay for infectious disease but also for other kinds of clinical tests:

- Set the target CV derived from simple one-way ANOVA model of specimen (for example, 10%). This value should be defined before the commencement of study.

- Design experiment to evaluate between- and within-individual error.

- Conduct experiment.

- Analyze data with ANOVA model with and without heterogeneous error variance.

- If CV exceeds the target, review the operating procedures.

- Conduct experiment a second time.

- Consequently analyze data to ascertain any improvement.

- Return to step 5 until CV becomes less than the target.

Table 7: Difference in estimated overall within-individual error between the two trials.

	Estimated overall error variance (ε_{ijk})	
Condition	1st trial	2nd trial*
Nil	1.3866	0.000025
TB-Antigen	6.9003	0.000062
Mitogen	35.3152	1.0814

*$P < 0.0001$

In-house quality control for effective transfer of skills is a topic of interest in our proposal and this should be carried out easily, at a low cost, whilst assuring objective and quantitative assessment in a clinical laboratory where resources such as reagents, manpower and feasibility of sample collection are limited. Our plan meets the above requirement. Measurements could be sent via the internet and analyzed in a statistical way by a joint-research facility inside or outside the country and an immediate feedback should be sent in an appropriate manner. Such continuous efforts to share information are important to maintain quality levels over a long distance [19].

In this age of evidence-based medicine and development of new diagnostic technologies, quality of laboratory tests is essential. There is an urgent need for validation and standardization of the new assays before they are adopted into clinical diagnostics [20]. Until such time as an effective quality control system is established, our approach is valuable to assure the quality of laboratory tests for timely diagnosis and treatment of infectious diseases. Another favorable design or analytical method might be suggested by others in the future studies, seeing that no standard way of quality monitoring has been proposed so far. We expect that the successful experience gathered in the present study will provide useful information for further comparison and discussion.

Our study has some limitations. It was obvious that outliers influenced statistical analysis in the first trial and exact causes of error in each condition were not clearly specified by the present analysis itself [15]. Through repeated experiments, the causes of error might be clearer, although all errors in our study decreased dramatically after a single intervention. We should also emphasize in conclusion, that a number of procedures should be combined to establish a total quality assurance system.

Conclusion

In a setting where a modern quality control system has not been entirely established, a laboratory test could be assessed quantitatively and such objective assessment is helpful for quality improvement of the test, if an appropriate design of experiment and statistical method are chosen. The design of experiment BIBD and analytical models for ANOVA were useful for objective assessment of individual skills in each stage of a multi-step immunoassay for tuberculosis in a laboratory with limited resources. A proposed plan to assess the level of proficiency might be useful for skill improvement of clinical testing especially for infectious diseases when monitoring is difficult to assure the sustainability of the technology transferred.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NTLH participated in supervising the on-site implementation of the study, drafting the paper or substantially revising it. NI was responsible for making conception, design and overall supervision of the study, analysis and interpretation of data, drafting the paper or substantially revising it. NK participated in making conception and design of the study, analysis and interpretation of data, drafting the paper or substantially revising it. LTH carried out the immunoassays. DBT participated in on-site implementation. VTXT carried out the immunoassays. IM participated in technical transfer and supervision. NH was responsible for technical transfer and supervision. KH was responsible for technical transfer and supervision. SS participated in conception and design of the study. LTL participated in conception, design and supervision of the study. All authors read and approved the manuscript.

Acknowledgements

The authors thank Dr. Hiroshi Ohara (IMCJ-BMH Medical Collaboration Center) and Dr. Pham Huu Thuong (Hanoi Tuberculosis and Lung Disease Hospital) for supporting management. The authors also thank Kazuko Tanabe D.V.M. for her critical reading of this manuscript. This work was supported by grants from the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT and International Cooperation Research Grant, the Ministry of Health, Labor and Welfare, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

References

1. Westgard JO: **Basic Planning for Quality.** Madison: Westgard QC Inc; 2000.
2. Kazmierczak SC: **Laboratory quality control: using patient data to assess analytical performance.** *Clin Chem Lab Med* 2003, **41**:617-27.
3. Westgard JO, Barry PL, Hunt MR, Groth T: **A multi-rule Shewhart chart for quality control in clinical chemistry.** *Clin Chem* 1981, **27**:493-501.
4. Heuck CC, Deom A: **Healthcare in the developing world: Need for appropriate laboratory technology.** *Clin Chem* 1991, **37**:490-6.
5. Smith PG, Morrow RH: **Field trials of health interventions in developing countries: A toolbox.** 2nd edition. Oxford: Macmillan Press Limited; 1996.
6. Peruski AH, Peruski LF Jr: **Immunological methods for detection and identification of infectious disease and biological warfare agents.** *Clin Diagn Lab Immunol* 2003, **10**:506-13.
7. Peeling RW, Mabey D, Herring A, Hook EW 3rd: **Why do we need quality-assured diagnostic tests for sexually transmitted infections.** *Nat Rev Microbiol* 2006, **4**:S7-19.
8. Lock RJ: **My approach to internal quality control in a clinical immunology laboratory.** *J Clin Pathol* 2006, **59**:681-684.
9. Pai M, Riley LW, Colford JM Jr: **Interferon-gamma assays in the immunodiagnosis of tuberculosis: a systematic review.** *Lancet Infect Dis* 2004, **4**:761-76.
10. Armitage P, Berry G, Matthews JNS: **Statistical method in medical research.** 4th edition. Malden: Blackwell science Ltd; 2002.
11. Williams GW, Schork MA: **Basic statistics for quality control in the clinical laboratory.** *Crit Rev Clin Lab Sci* 1982, **17**:171-99.
12. Fleiss JL: **The design and analysis of clinical experiments.** New York: John Wiley & Sons, Inc; 1999.
13. Endres HG, Hucke C, Holland-Letz T, Trampisch HJ: **A new efficient trial design for assessing reliability of ankle-brachial index measures by three different observer groups.** *BMC Cardiovasc Disord* 2006, **6**:33.
14. Varma AAO, Chilton NW, Kutscher AH: **Studies in the Design and Analysis of Dental Experiments: 13. Balanced Incomplete Block Design.** *J Dent Res* 1970, **49**:1166-1171.
15. Marks V: **False-positive immunoassay results: a multicenter survey of erroneous immunoassay results from assays of 74 analytes in 10 donors from 66 laboratories in seven countries.** *Clin Chem* 2002, **48**:2008-16.
16. Ricós C, García-Victoria M, de la Fuente B: **Quality indicators and specifications for the extra-analytical phases in clinical laboratory management.** *Clin Chem Lab Med* 2004, **42**:578-82.
17. Cellestis Inc: *QuantiFERON Precision and reproducibility report* [http://www.cellestis.com/IRM/content/gold/Precisionproductivity.pdf]. Accessed 18 October 2008.
18. Menzies D, Pai M, Comstock G: **Meta-analysis: new tests for the diagnosis of latent tuberculosis infection: areas of uncertainty and recommendations for research.** *Ann Intern Med* 2007, **146**:340-54.
19. Coloma J, Harris E: **Sustainable transfer of biotechnology to developing countries: fighting poverty by bringing scientific tools to developing-country partners.** *Ann N Y Acad Sci* 2008, **1136**:358-68.
20. Lalvani A, Meroni PL, Millington KA, Modolo ML, Plebani M, Tincani A, Villalta D, Doria A, Ghirardello A: **Recent advances in diagnostic technology: applications in autoimmune and infectious diseases.** *Clin Exp Rheumatol* 2008, **26(1 Suppl 48)**:S62-6.

Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-2334/9/66/prepub

Human Immunology 70 (2009) 527–531

Contents lists available at ScienceDirect

ASHI American Society for Histocompatibility & Immunogenetics

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

Human Immunology

Association of human leukocyte antigen class II alleles with severe acute respiratory syndrome in the Vietnamese population

Naoto Keicho [a,*], Satoru Itoyama [a], Koichi Kashiwase [a], Nguyen Chi Phi [c], Hoang Thuy Long [d], Le Dang Ha [e], Vo Van Ban [f], Bach Khanh Hoa [g], Nguyen Thi Le Hang [h], Minako Hijikata [a], Shinsaku Sakurada [a], Masahiro Satake [b], Katsushi Tokunaga [i], Takehiko Sasazuki [j], Tran Quy [c]

[a] Department of Respiratory Diseases, Research Institute, Tokyo, Japan
[b] Japanese Red Cross Tokyo Metropolitan Blood Center, Tokyo, Japan
[c] Bach Mai Hospital, Hanoi, Viet Nam
[d] National Institute of Hygiene and Epidemiology, Hanoi, Viet Nam
[e] Institute for Clinical Research in Tropical Medicine, Hanoi, Viet Nam
[f] Hanoi-French Hospital, Hanoi, Viet Nam
[g] Faculty of Hematology and Blood Transfusion, Hanoi Medical School, Hanoi, Viet Nam
[h] IMCJ-BMH Medical Collaboration Center, Hanoi, Viet Nam
[i] Department of Human Genetics, Graduate School of Medicine, University of Tokyo, Tokyo, Japan
[j] International Medical Center of Japan, Tokyo, Japan

ARTICLE INFO

Article history:
Received 27 January 2009
Accepted 8 May 2009
Available online 13 May 2009

Keywords:
Severe acute respiratory syndrome (SARS)
Human leukocyte antigen (HLA)
Association study
Genetic polymorphism
Viet Nam

ABSTRACT

Excessive immune response is believed to play a role in the development of severe acute respiratory syndrome (SARS). Inhomogeneous spread of SARS led one to think of an Asian genetic predisposition and contribution of human leukocyte antigen (HLA) to the disease susceptibility. However, past case-control studies showed inconsistent results. In Viet Nam, of 62 patients with SARS, 44 participated in the present study together with 103 individuals who had contact with SARS patients and 50 without contact history. HLA-DRB1*12 was more frequently shown in SARS patients than in controls (corrected $p = 0.042$). HLA-DRB1*1202, the predominant allele in the Vietnamese population showed the strongest association with SARS in a dominant model (corrected $p = 0.0065$ and 0.0052, depending on the controls to be compared). Our results and accumulated data on HLA in the Asian populations would help in the understanding of associations with emerging infectious diseases.

1. Introduction

Severe acute respiratory syndrome (SARS) originated in southern China in November 2002, reaching Hong Kong in February 2003 and thereafter spreading rapidly to other countries in Asia, Europe, and North America; it ended by July 2003 [1]. In total, 8098 individuals had SARS worldwide, with the majority of the patients confined to regions around southeastern or eastern Asia (Mainland Chinese, Hong Kong residents, Vietnamese, Singaporeans, and Taiwanese), which raised the question as to the possibility of an Asian-specific genetic predisposition to SARS [2–5].

This emerging disease was caused by a novel coronavirus (SARS-CoV) and was characterized by extensive inflammatory damage of alveolar epithelium in the lung, resulting in death in 10% of the patients. Because the lung lesions develop approximately 1 week after the peak of viral replication in the lung, hyperimmune response has been believed to play a role in the progress of the disease, although details of the immunologic mechanism and effective therapeutic measures for acute lung injury caused by emerging viruses such as SARS-CoV and H5N1 remain unknown [6–8].

Human leukocyte antigen (HLA) variations are often associated with susceptibility or resistance to a wide range of infections, including malaria, tuberculosis, leprosy, HIV and virus-induced hepatitis [9,10]. In this context, HLA was the first human gene to be investigated immediately after the SARS outbreak. However, such reports from Taiwan [2,4], a study in Hong Kong [3], and a study in mainland China [5] showed disease association of different alleles and no consensus has been reached yet for interpretation of the overall data.

In the present study, we presented genotyping data of HLA class I and class II genes in Vietnamese SARS patients and controls, after which we gained insight into the overall association studies relating to HLA allele and haplotype distribution in Asian populations.

2. Subjects and methods

2.1. Subjects

This study was reviewed and approved by ethics committees in the Ministry of Health in Viet Nam as well as the International

Naoto Keicho and Satoru Itoyama contributed equally to this work.

* Corresponding author.
E-mail address: nkeicho-tky@umin.ac.jp (N. Keicho).

0198-8859/09/$32.00 - see front matter © 2009 American Society for Histocompatibility and Immunogenetics. Published by Elsevier Inc. All rights reserved.
doi:10.1016/j.humimm.2009.05.006

Medical Center in Japan. Written informed consent had been obtained from all subjects, and detailed demographics of the subjects had been described beforehand [11]. In brief terms, the study population comprised 44 SARS patients, 103 staff members of the same hospital as control subjects who had come into contact with SARS patients but had not developed SARS, and 50 healthy individuals having had no contact history with SARS patients. All participants were unrelated Vietnamese. No samples from deceased patients were available for this study.

2.2. HLA typing

Genomic DNA was extracted from the whole blood by using the QIAampTM DNA Blood Midi Kit (Qiagen Sciences, Germantown, MD). Plasma samples six months on average after the outbreak were tested for anti-SARS-CoV antibodies by SARS ELISA (Genelabs Diagnostics Pty Ltd, Singapore) from all participants [12]. DNA-based HLA typing was performed by Luminex Multi-Analyte Profiling system (xMAP) with WAKFlow HLA typing kit (Wakunaga, Hiroshima, Japan) as described elsewhere [13]. Briefly, highly polymorphic exons 2 and 3 of HLA-A, -B, and -C genes and exon 2 of HLA-DRB1 and -DQB1 genes were amplified using primer pairs attached to the kit. Each PCR product was hybridized with sequence-specific oligonucleotide probes, complementary to the allele-specific sequences. Reproducibility was checked between two independent measurements of randomly chosen samples and the level of agreement was more than 99% (183/184 alleles). Samples showing ambiguous patterns were subjected to sequence-based typing by using AlleleSEQR HLA typing kit (Abbott JAPAN, Tokyo, Japan) and analyzed on an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

2.3. Statistical analysis

Disease association was assessed by the χ^2 test. When any expected number in the 2×2 contingency table was less than 5, the p value was directly calculated by Fisher's exact test. Values of $p < 0.05$ are shown. Corrected p values (p_c), p values multiplied by the number of comparisons in each locus, are also shown. Values of $p_c < 0.05$ were considered to be statistically significant.

3. Results

Of 62 patient cases corresponding to the World Health Organization case definition of probable SARS, five patients died and another three were not Vietnamese. As a result, 44 of the remaining 54 SARS patients after recovery (Cases), 103 individuals who had contact with SARS patients (Contacts) and 50 without contact (No contacts) were included within this study. DNA samples from two of 103 Contacts showed inconclusive genotyping results and were excluded from the present analysis.

We primarily compared the Cases with Contacts, No contacts, or both. Subsequently all Cases and 16 of the 101 Contacts were revealed to have anti-SARS-CoV antibodies in their blood and we secondarily analyzed them together with SARS patients as the Infected group ($n = 44 + 16$) and compared with the Uninfected group ($n = 101 - 16$). Allele frequencies of HLA class I and class II genes in each group were first represented by low resolution (two-digit) typing and are listed in Tables 1 and 2, respectively. When the number of comparisons was considered at each locus, HLA class I genes did not show any significant association ($p_c > 0.10$). On the other hand, among HLA class II genes, HLA-DRB1*12 showed positive association ($p = 0.0032$, $p_c = 0.042$), and HLA-DRB1*13 showed marginally negative association ($p = 0.0069$, $pc = 0.090$) when the SARS group was compared with the Contacts. A similar tendency was observed when the SARS group was compared with the No-contacts as well. However, the same alleles did not show any strong association when the Infected group was compared with Uninfected.

HLA-DRB1*1202 allele was the only allele of HLA-DRB1*12 identified in this population by higher resolution (four-digit) typing (Table 3). When the Contacts and No-contacts were collected together as a single control group, HLA-DRB1*1202 showed further significant association ($p = 0.0011$, $p_c = 0.014$, data not shown). In our previous population-based study on Vietnamese HLA alleles, the frequency of HLA-DRB1*1202 allele in 170 healthy Hanoi citizens was 0.353 [13]. The frequency of HLA-DRB1*1202 in our SARS patients (0.466) remained higher than that of the general population in Hanoi. Under the dominant model, HLA-DRB1*1202 was the most strongly associated with SARS as shown in Table 4 ($p_c = 0.0065$ and 0.0052 depending on the controls to be compared).

HLA-DRB1*13 was composed of DRB1*1301, *1302 and *1303 but no single alleles were further associated with the disease (Table 3). HLA association between the Infected and Uninfected groups did not exceed any association of HLA alleles observed between the Cases and Contacts (Tables 1 and 2). Possible association of HLA polymorphism with severity of SARS was not investigated in this study because of the small numbers in each subgroup. No significant deviation from Hardy-Weinberg equilibrium was observed in the control populations ($p > 0.10$).

4. Discussion

No particular alleles of class I genes were associated with SARS in the Vietnamese population, whereas HLA-DRB1*1202 showed a significant association with SARS development. In Viet Nam, the majority of the patients were health care staff in one hospital, and our study was the only HLA report from this country.

As far as we know, at the present time HLA reports regarding association with SARS [2–5] have been published in Taiwan, Hong Kong, and southern China. Interestingly, all reports showed inconsistent results, although HLA patterns in the general population of their countries were quite similar, presumably attributable to rather homogenous ancestral gene pool around southern China. It is known that ethnicity of the Vietnamese is also influenced by the population resident in southern China in the Bronze Age [13]. Thus, comparative consideration of the previous four association studies in these Asian populations is worthwhile.

First of all, our data can be interpreted naturally and consistently with a recent large study from Southern China [5], which showed an increase in frequency of several HLA-A and -B antigens and DRB1*12 allele, although the alleles did not reach significant levels when multiple comparisons were made. In case of HLA-DRB1*12, allele frequency was 32.6% in 95 SARS and 22.8% in 403 controls with a value of $p < 0.046$ in their study [5]. We were able to confirm this disease association of HLA-DRB1*12 clearly in the present study.

In the Vietnamese population, the frequency of HLA-DRB1*1202 mainly consisting of DRB1*12 is notably higher than in the Southern Chinese population [13] and therefore the HLA association with SARS might have been more obvious even in the smaller sample study like ours. In particular, our advantage is that the majority of cases and controls were exposed to SARS-CoV during a rather short period inside one hospital through a single spread from Hong Kong [14]. Such homogeneity of environmental and pathogenic factors might have provided a favorable situation to identify host genetic factors without detailed consideration of unknown confounders.

Another recent Taiwanese genome-epidemiologic study demonstrated potential association of HLA-Cw*0801 with SARS infection between seropositive and seronegative cases [4]. In fact, even in our study, HLA-Cw*0801 was more frequently observed in the Infected group (24.2%) than in the Uninfected group (17.1%), although not reaching a significant level, probably because of insufficient statistical power. More interestingly, a five-locus haplotype including both DRB1*1202 and Cw*0801 alleles, A*1101-Cw*0801-B*1502-DRB1*1202-DQB1*0301, is the most frequent haplotype in

Table 1
Frequencies of HLA class I alleles in SARS cases and controls (two-digit typing)

	Cases		Contacts		p Value	No contacts		p Value		Cases+CtAb(+)		CtAb(−)		p Value
	2n = 88		2n = 202			2n = 100				2n = 120		2n = 170		
A*01	3	3.4%	4	2.0%		3	3.0%		A*01	3	2.5%	4	2.4%	
A*02	19	21.6%	46	22.8%		25	25.0%		A*02	31	25.8%	34	20.0%	
A*03	0	0.0%	2	1.0%		1	1.0%		A*03	0	0.0%	2	1.2%	
A*11	31	35.2%	57	28.2%		33	33.0%		A*11	37	30.8%	51	30.0%	
A*24	14	15.9%	32	15.8%		13	13.0%		A*24	19	15.8%	27	15.9%	
A*26	0	0.0%	4	2.0%		3	3.0%		A*26	0	0.0%	4	2.4%	
A*29	5	5.7%	19	9.4%		8	8.0%		A*29	10	8.3%	14	8.2%	
A*30	0	0.0%	1	0.5%		0	0.0%		A*30	0	0.0%	1	0.6%	
A*31	2	2.3%	1	0.5%		2	2.0%		A*31	2	1.7%	1	0.6%	
A*32	0	0.0%	1	0.5%		0	0.0%		A*32	0	0.0%	1	0.6%	
A*33	12	13.6%	33	16.3%		10	10.0%		A*33	16	13.3%	29	17.1%	
A*34	1	1.1%	1	0.5%		1	1.0%		A*34	1	0.8%	1	0.6%	
A*68	1	1.1%	0	0.0%		0	0.0%		A*68	1	0.8%	0	0.0%	
A*74	0	0.0%	1	0.5%		1	1.0%		A*74	0	0.0%	1	0.6%	
B*07	7	8.0%	22	10.9%		9	9.0%		B*07	11	9.2%	18	10.6%	
B*13	3	3.4%	6	3.0%		9	9.0%		B*13	4	3.3%	5	2.9%	
B*15	27	30.7%	53	26.2%		25	25.0%		B*15	38	31.7%	42	24.7%	
B*18	2	2.3%	6	3.0%		1	1.0%		B*18	2	1.7%	6	3.5%	
B*27	0	0.0%	5	2.5%		2	2.0%		B*27	0	0.0%	5	2.9%	
B*35	5	5.7%	10	5.0%		2	2.0%		B*35	7	5.8%	8	4.7%	
B*37	0	0.0%	2	1.0%		1	1.0%		B*37	0	0.0%	2	1.2%	
B*38	6	6.8%	15	7.4%		7	7.0%		B*38	8	6.7%	13	7.6%	
B*39	2	2.3%	4	2.0%		1	1.0%		B*39	2	1.7%	4	2.4%	
B*40	3	3.4%	11	5.4%		6	6.0%		B*40	5	4.2%	9	5.3%	
B*41	1	1.1%	0	0.0%		0	0.0%		B*41	1	0.8%	0	0.0%	
B*44	4	4.5%	9	4.5%		3	3.0%		B*44	4	3.3%	9	5.3%	
B*46	7	8.0%	20	9.9%		11	11.0%		B*46	11	9.2%	16	9.4%	
B*48	**5**	**5.7%**	**2**	**1.0%**	**0.0286**	**0**	**0.0%**	**0.0211**	B*48	6	5.0%	**1**	**0.6%**	**0.0214**
B*49	0	0.0%	1	0.5%		0	0.0%		B*49	0	0.0%	1	0.6%	
B*51	**1**	**1.1%**	4	2.0%		**8**	**8.0%**	**0.0380**	B*51	1	0.8%	4	2.4%	
B*52	0	0.0%	0	0.0%		3	3.0%		B*52	0	0.0%	0	0.0%	
B*54	4	4.5%	5	2.5%		3	3.0%		B*54	5	4.2%	4	2.4%	
B*55	0	0.0%	2	1.0%		1	1.0%		B*55	0	0.0%	2	1.2%	
B*56	2	2.3%	3	1.5%		0	0.0%		B*56	3	2.5%	2	1.2%	
B*57	3	3.4%	5	2.5%		2	2.0%		B*57	3	2.5%	5	2.9%	
B*58	6	6.8%	17	8.4%		6	6.0%		B*58	9	7.5%	14	8.2%	
Cw*01	12	13.6%	28	13.9%		13	13.0%		Cw*01	18	15.0%	22	12.9%	
Cw*03	13	14.8%	37	18.3%		21	21.0%		Cw*03	19	15.8%	31	18.2%	
Cw*04	10	11.4%	18	8.9%		7	7.0%		Cw*04	14	11.7%	14	8.2%	
Cw*05	0	0.0%	0	0.0%		1	1.0%		Cw*05	0	0.0%	0	0.0%	
Cw*06	3	3.4%	8	4.0%		3	3.0%		Cw*06	3	2.5%	8	4.7%	
Cw*07	19	21.6%	46	22.8%		17	17.0%		Cw*07	22	18.3%	43	25.3%	
Cw*08	22	25.0%	36	17.8%		18	18.0%		Cw*08	29	24.2%	29	17.1%	
Cw*12	1	1.1%	4	2.0%		3	3.0%		Cw*12	2	1.7%	3	1.8%	
Cw*14	0	0.0%	2	1.0%		4	4.0%		Cw*14	0	0.0%	2	1.2%	
Cw*15	7	8.0%	22	10.9%		13	13.0%		Cw*15	12	10.0%	17	10.0%	
Cw*16	0	0.0%	1	0.5%		0	0.0%		Cw*16	0	0.0%	1	0.6%	
Cw*17	1	1.1%	0	0.0%		0	0.0%		Cw*17	1	0.8%	0	0.0%	

Cases = 44 SARS patients.
Contacts = 101 individuals with contact with SARS patients.
No contacts = 50 individuals without contact.
Cases+CtAb(+) = 44 SARS patients together with 16 contacts with anti-SARS-CoV antibodies.
CtAb(−) = 85 contacts without anti-SARS-CoV antibodies.
Uncorrected *p* values <0.05 are shown in boldface type.

the Vietnamese population [13]. This implies the above haplotype might widely confer disease susceptibility among Asians, because alleles carried by this haplotype are rather common in Asians including the Southern Chinese.

On the other hand, the protective effect of HLA-DRB1*13 against SARS found in our study might be rather weak and, for the time being, difficult to be supported by other studies, although this association itself is interesting because HLA-DRB1*13 has been reported to play a protective role in HBV infection [15,16] and malaria [17]. The possibly resistant allele, HLA-DRB1*13 is also one of the characteristic alleles in the Korean and Japanese population that did not experience SARS [18].

Selective forces to particular HLA-DRB1 alleles (*e.g.*, conferring resistance to bacterial pathogens) other than a balancing selection have been discussed in Pacific/Asian populations including Vietnamese [13,19]. Resultant limited variation of their HLA repertoires may be disadvantageous to protection against emerging infections. On the other hand, it is also conceivable that such a common Asian allele or haplotype may evoke an unfavorable immune reaction to new pathogens in the disease progression. Strong association of HLA-DRB1*1202 in a dominant model might support the latter possibility. Although HLA alleles probably account for only a part of disease susceptibility, this hypothesis should be carefully tested in the recent critical circumstances of the prevailing human infection caused by avian influenza H5N1 in almost the same area of southeast Asia [20].

Both CD4+ and CD8+ T cell responses to the epitopes from SARS-CoV have been observed in the peripheral blood of patients [21]. HLA class II–restricted T cell responses have been investigated in SARS-CoV as well as other viruses and may be important in

Table 2
Frequencies of HLA class II alleles in SARS and controls (two-digit typing)

	Cases		Contacts		p Value	No contacts		p Value		Cases+CtAb(+)		CtAb(−)		p Value
	2n = 88		2n = 202			2n = 100				2n = 120		2n = 170		
DRB1*01	0	0.0%	1	0.5%		0	0.0%		DRB1*01	0	0.0%	1	0.6%	
DRB1*03	6	6.8%	14	6.9%		2	2.0%		DRB1*03	8	6.7%	12	7.1%	
DRB1*04	**2**	**2.3%**	**18**	**8.9%**	**0.0403**	**12**	**12.0%**	**0.0112**	**DRB1*04**	**3**	**2.5%**	**17**	**10.0%**	**0.0130**
DRB1*07	5	5.7%	15	7.4%		5	5.0%		DRB1*07	5	4.2%	15	8.8%	
DRB1*08	**1**	**1.1%**	6	3.0%		**9**	**9.0%**	**0.0207**	DRB1*08	4	3.3%	3	1.8%	
DRB1*09	6	6.8%	26	12.9%		10	10.0%		DRB1*09	12	10.0%	20	11.8%	
DRB1*10	5	5.7%	15	7.4%		8	8.0%		DRB1*10	7	5.8%	13	7.6%	
DRB1*11	2	2.3%	5	2.5%		1	1.0%		DRB1*11	2	1.7%	5	2.9%	
DRB1*12	**41**	**46.6%**	**58**	**28.7%**	**0.0032**	**27**	**27.0%**	**0.0053**	**DRB1*12**	**50**	**41.7%**	**49**	**28.8%**	**0.0231**
DRB1*13	**0**	**0.0%**	**15**	**7.4%**	**0.0069**	**6**	**6.0%**	**0.0306**	DRB1*13	4	3.3%	11	6.5%	
DRB1*14	9	10.2%	8	4.0%		7	7.0%		DRB1*14	11	9.2%	6	3.5%	
DRB1*15	11	12.5%	20	9.9%		9	9.0%		DRB1*15	14	11.7%	17	10.0%	
DRB1*16	0	0.0%	1	0.5%		4	4.0%		DRB1*16	0	0.0%	1	0.6%	
DQB1*02	11	12.5%	23	11.4%		5	5.0%		DQB1*02	13	11.0%	21	12.4%	
DQB1*03	49	55.7%	109	54.0%		51	51.0		%DQB1*03	66	55.9%	92	54.1%	
DQB1*04	1	1.1%	8	4.0%		4	4.0%		DQB1*04	2	1.7%	7	4.1%	
DQB1*05	23	26.1%	39	19.3%		26	26.0%		DQB1*05	29	24.6%	33	19.4%	
DQB1*06	**4**	**4.5%**	23	11.4%		**14**	**14.0%**	**0.0279**	DQB1*06	10	8.5%	17	10.0%	

Cases = 44 SARS patients.
Contacts = 101 individuals with contact with SARS patients.
No contacts = 50 individuals without contact.
Cases+CtAb(+) = 44 SARS patients together with 16 contacts with anti-SARS-CoV antibodies.
CtAb(−) = 85 contacts without anti-SARS-CoV antibodies.
Uncorrected *p* values <0.05 are shown in boldface type.

immunologic control against SARS [22, 23]. Our finding that association of the above HLA class II alleles was stronger between Cases and Contacts than between Infected and Uninfected might also support its role in progress of the disease after infection, rather than in transmission of the virus to the host. Future investigation of the host-pathogen interaction is awaited.

Our report and the above-mentioned articles [4,5] did not support the findings of the remaining previous reports [2,3]. The first one showed an association of HLA-B*46 and B*54 with development or severity of SARS in Taiwan [2]. The investigators analyzed 33 probable SARS patients, but this association was not significant when multiple testing was taken into consideration in their study. Further subgroup analysis of six severe or deceased cases should be carefully interpreted in general. Also, results from our study and three other studies could not reproduce the report from Hong Kong [3]. HLA-B*0703 showing positive association but not commonly observed in Asian populations including Hong Kong Chinese. Instead, B*0702 or B*0705 are known to be major alleles of the HLA-B7 serotype in Asia [24]. HLA-DRB1*0301 allele negatively associated with SARS in their study, is not so frequently seen in the Asian populations either [25], but observed more commonly in European or African descent. Even a small percentage of mixed ethnicity in the control population should be carefully assessed, because the analysis depends partly on a relatively unbalanced number of cases (n = 87) and controls (n = 18,774).

A major weakness of our own study is the limited number of cases similar to those in other studies. Although our conclusion here derives from the most rational deduction based on accumulated results of each small study, different associations in other studies could also be interpreted in other ways, for instance, by the interaction with other environmental or pathogenic factors such as possible difference of mutation in SARS-CoV. Alternatively, it is also possible that the HLA-genes are merely markers of the disease susceptibility and that other immune-related genes in the HLA regions may be more deeply involved in the risk of disease. Genetic contribution of non-HLA immune molecules has also been studied extensively even now [6–8]. We should collect all information and prepare for the threat of a future outbreak of emerging diseases such as H5N1 infection that causes lethal acute lung injury presumably through a similar immune mechanism.

Table 3
Frequencies of HLA class I or II alleles showing significant association in previous reports and the present study on SARS

Two-digit allele	Four-digit allele	Cases		Contacts		p Value	No contacts		p Value		Cases+CtAb(+)		CtAb(−)		p Value
		2n = 88		2n = 202			2n = 100				2n = 120		2n = 170		
B*07	B*0702	1	1.1%	3	1.5%		0	0.0%		B*0702	1	0.8%	3	1.8%	
	B*0705	6	6.8%	19	9.4%		9	9.0%		B*0705	10	8.3%	15	8.8%	
B*46	B*4601	7	8.0%	20	9.9%		11	11.0%		B*4601	11	9.2%	16	9.4%	
B*54	B*5401	4	4.5%	5	2.5%		3	3.0%		B*5401	5	4.2%	4	2.4%	
Cw*08	Cw*0801	22	25.0%	36	17.8%		18	18.0%		Cw*0801	29	24.2%	29	17.1%	
DRB1*03	DRB1*0301	6	6.8%	14	6.9%		2	2.0%		DRB1*0301	8	6.7%	12	7.1%	
DRB1*12	**DRB1*1202**	**41**	**46.6%**	**58**	**28.7%**	**0.0032**	**27**	**27.0%**	**0.0053**	**DRB1*1202**	**50**	**41.7%**	**49**	**28.8%**	**0.0231**
DRB1*13	DRB1*1301	0	0.0%	2	1.0%		1	1.0%		DRB1*1301	0	0.0%	2	1.2%	
	DRB1*1302	0	0.0%	5	2.5%		4	4.0%		DRB1*1302	1	0.8%	4	2.4%	
	DRB1*1303	0	0.0%	8	4.0%		1	1.0%		DRB1*1303	3	2.5%	5	2.9%	

Cases = 44 SARS patients.
Contacts = 101 individuals with contact with SARS patients.
No contacts = 50 individuals without contact.
Cases+CtAb(+) = 44 SARS patients together with 16 contacts with anti-SARS-CoV antibodies.
CtAb(−) = 85 contacts without anti-SARS-CoV antibodies.
Uncorrected *p* values <0.05 are shown in boldface type.

Table 4
Genotype pattern of HLA-DRB1*1202 and association with SARS

Model	Cases		Contacts		p Value	p_c	No contacts		p Value	p_c
	n = 44		n = 101				n =50			
DRB1*1202										
Recessive										
+/+	6	13.6%	9	8.9%			5	10.0%		
+/− or −/−	38	86.4%	92	91.1%			45	90.0%		
Dominant										
+/+ or +/−	**35**	**79.5%**	**49**	**48.5%**	**0.0005**	**0.0065**	**22**	**44.0%**	**0.0004**	**0.0052**
−/−	**9**	**20.5%**	**52**	**51.5%**			**28**	**56.0%**		

Cases = 44 SARS patients.
Contacts = 101 individuals with contact with SARS patients.
No contacts = 50 individuals without contact.
Uncorrected *p* values <0.05 are shown in boldface type.

In conclusion, our study demonstrated that an HLA class II allele, HLA-DRB1*1202 is a new candidate allele involved in the progress of SARS, which enabled us to evaluate previous HLA studies on the basis of alleles and haplotypes common to the Asian populations. Accumulation of these studies would also help when planning a future effective vaccination strategy.

Acknowledgments

The authors thank Dr. Masaki Matsushita (Wakunaga Pharmaceutical Co., Ltd), Ms. Mutsuko Minemoto, and Mr. Takahiro Ichihara (Japanese Red Cross Tokyo Metropolitan Blood Center) for technical support of HLA typing. The authors also thank Ms. Pham Thi Phuong Thuy, and Ms. Nguyen Thi Thu Ha for their help in the management and coordination of this study in Viet Nam. Finally, the authors thank Kazuko Tanabe, D.V.M., and Mr. John Crosskey for their critical reading of this manuscript. This work was supported by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT Japan.

References

[1] Peiris JS, Guan Y, Yuen KY. Severe acute respiratory syndrome. Nat Med 2004; 10:S88–97.
[2] Lin M, Tseng HK, Trejaut JA, Lee HL, Loo JH, Chu CC, et al. Association of HLA class I with severe acute respiratory syndrome coronavirus infection. BMC Med Genet 2003;4:9.
[3] Ng MH, Lau KM, Li L, Cheng SH, Chan WY, Hui PK, et al. Association of human-leukocyte-antigen class I (B*0703) and class II (DRB1*0301) genotypes with susceptibility and resistance to the development of severe acute respiratory syndrome. J Infect Dis 2004;190:515–8.
[4] Chen YM, Liang SY, Shih YP, Chen CY, Lee YM, Chang L, et al. Epidemiological and genetic correlates of severe acute respiratory syndrome coronavirus infection in the hospital with the highest nosocomial infection rate in Taiwan in 2003. J Clin Microbiol 2006;44:359–65.
[5] Xiong P, Zeng X, Song MS, Jia SW, Zhong MH, Xiao LL, et al. Lack of association between HLA-A, -B and -DRB1 alleles and the development of SARS: A cohort of 95 SARS-recovered individuals in a population of Guangdong, southern China. Int J Immunogenet 2008;35:69–74.
[6] Gu J, Korteweg C. Pathology and pathogenesis of severe acute respiratory syndrome. Am J Pathol 2007;170:1136–47.
[7] Lau YL, Peiris JS. Pathogenesis of severe acute respiratory syndrome. Curr Opin Immunol 2005;17:404–10.
[8] Chen J, Subbarao K. The immunobiology of SARS. Annu Rev Immunol 2007;25: 443–72.
[9] Hill AV. The immunogenetics of human infectious diseases. Annu Rev Immunol 1998;16:593–617.
[10] Martin MP, Carrington M. Immunogenetics of viral infections. Curr Opin Immunol 2005;17:510–6.
[11] Itoyama S, Keicho N, Quy T, Phi NC, Long HT, Ha le D, et al. ACE1 polymorphism and progression of SARS. Biochem Biophys Res Commun 2004;323:1124–9.
[12] Hamano E, Hijikata M, Itoyama S, Quy T, Phi NC, Long HT, et al. Polymorphisms of interferon-inducible genes OAS-1 and MxA associated with SARS in the Vietnamese population. Biochem Biophys Res Commun 2005;329:1234–9.
[13] Hoa BK, Hang NT, Kashiwase K, Ohashi J, Lien LT, Horie T, et al. HLA-A, -B, -C, -DRB1 and -DQB1 alleles and haplotypes in the Kinh population in Vietnam. Tissue Antigens 2008;71:127–34.
[14] Nishiura H, Kuratsuji T, Quy T, Phi NC, Van Ban V, Ha LE, et al. Rapid awareness and transmission of severe acute respiratory syndrome in Hanoi French Hospital, Vietnam. Am J Trop Med Hyg 2005;73:17–25.
[15] Hohler T, Gerken G, Notghi A, Lubjuhn R, Taheri H, Protzer U, et al. HLA-DRB1*1301 and *1302 protect against chronic hepatitis B. J Hepatol 1997;26: 503–7.
[16] Thursz MR, Kwiatkowski D, Allsopp CE, Greenwood BM, Thomas HC, Hill AV. Association between an MHC class II allele and clearance of hepatitis B virus in the Gambia. N Engl J Med 1995;332:1065–9.
[17] Hill AV, Allsopp CE, Kwiatkowski D, Anstey NM, Twumasi P, Rowe PA, et al. Common west African HLA antigens are associated with protection from severe malaria. Nature 1991;352:595–600.
[18] Park MH, Hwang YS, Park KS, Tokunaga K, Akaza T, Juji T, et al. HLA haplotypes in Koreans based on 107 families. Tissue Antigens 1998;51:347–55.
[19] Mack SJ, Bugawan TL, Moonsamy PV, Erlich JA, Trachtenberg EA, Paik YK, et al. Evolution of Pacific/Asian populations inferred from HLA class II allele frequency distributions. Tissue Antigens 2000;55:383–400.
[20] Abdel-Ghafar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, de Jong MD, et al. Update on avian influenza A (H5N1) virus infection in humans. Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza A (H5N1) Virus. N Engl J Med 2008;358:261–73.
[21] Li CK, Wu H, Yan H, Ma S, Wang L, Zhang M, et al. T cell responses to whole SARS coronavirus in humans. J Immunol 2008;181:5490–500.
[22] Libraty DH, O'Neil KM, Baker LM, Acosta LP, Olveda RM. Human CD4(+) memory T-lymphocyte responses to SARS coronavirus infection. Virology 2007;368:317–21.
[23] Yang J, James E, Roti M, Huston L, Gebe JA, Kwok WW. Searching immunodominant epitopes prior to epidemic: HLA class II-restricted SARS-CoV spike protein epitopes in unexposed individuals. Int Immunol 2009;21:63–71.
[24] Anthropology Search; Class I Allele Frequencies, Population Area: North-East Asia or South-East Asia. Available at: http://www.ncbi.nlm.nih.gov/projects/gv/mhc/ihwg.cgi?=ID=9&cmd=PRJOV. Accessed 28 May, 2009.
[25] Hardy S, Siauw PA, Scotland R, Gu W, Fajatin M, Luman S, et al. Chinese Hong Kong normal. In: Terasaki PI, Gjertson DW, ed. HLA 1997. Los Angeles: UCLA Tissue Typing Laboratory; 1997: 261.

Granulocyte/macrophage–colony-stimulating factor autoantibodies and myeloid cell immune functions in healthy subjects

Kanji Uchida,[1,2] Koh Nakata,[3] Takuji Suzuki,[1] Maurizio Luisetti,[4] Masato Watanabe,[3] Diana E. Koch,[1] Carrie A. Stevens,[5] David C. Beck,[1,6] Lee A. Denson,[7] Brenna C. Carey,[1] Naoto Keicho,[8] Jeffrey P. Krischer,[9] Yoshitsugu Yamada,[2] and Bruce C. Trapnell[1,6,10]

[1]Division of Pulmonary Biology, Cincinnati Children's Hospital Medical Center, OH; [2]Department of Anesthesiology, University of Tokyo, Tokyo, Japan; [3]Bioscience Medical Research Center, Niigata University Medical and Dental Hospital, Niigata, Japan; [4]Institute for Respiratory Disease, San Matteo Hospital Foundation for Research and Care, University of Pavia, Pavia, Italy; [5]Translational Research Trials Office, Cincinnati Children's Hospital Medical Center, OH; [6]Division of Pulmonary, Critical Care and Sleep Medicine, University of Cincinnati College of Medicine, OH; [7]Division of Gastroenterology, Cincinnati Children's Hospital Medical Center, OH; [8]Department of Respiratory Diseases, International Medical Center of Japan, Tokyo, Japan; [9]Division of Informatics and Biostatistics, University of South Florida, Tampa; and [10]Division of Pulmonary Medicine, Cincinnati Children's Hospital Medical Center, OH

High levels of granulocyte/macrophage–colony-stimulating factor (GM-CSF) autoantibodies are thought to cause pulmonary alveolar proteinosis (PAP), a rare syndrome characterized by myeloid dysfunction resulting in pulmonary surfactant accumulation and respiratory failure. Paradoxically, GM-CSF autoantibodies have been reported to occur rarely in healthy people and routinely in pharmaceutical intravenous immunoglobulin (IVIG) purified from serum pooled from healthy subjects. These findings suggest that either GM-CSF autoantibodies are normally present in healthy people at low levels that are difficult to detect or that serum pooled for IVIG purification may include asymptomatic persons with high levels of GM-CSF autoantibodies. Using several experimental approaches, GM-CSF autoantibodies were detected in all healthy subjects evaluated (n = 72) at low levels sufficient to rheostatically regulate multiple myeloid functions. Serum GM-CSF was more abundant than previously reported, but more than 99% was bound and neutralized by GM-CSF autoantibody. The critical threshold of GM-CSF autoantibodies associated with the development of PAP was determined. Results demonstrate that free serum GM-CSF is tightly maintained at low levels, identify a novel potential mechanism of innate immune regulation, help define the therapeutic window for potential clinical use of GM-CSF autoantibodies to treat inflammatory and autoimmune diseases, and have implications for the pathogenesis of PAP. (Blood. 2009;113:2547-2556)

Introduction

Granulocyte/macrophage–colony-stimulating factor (GM-CSF) is a pleiotropic cytokine regulator of myeloid and other immune and nonimmune cells that is required for terminal differentiation of alveolar macrophages in the lungs and regulates the basal functional capacity of circulating neutrophils in mice and humans.[1-7] The paracrine,[3,8] autocrine,[9] and endocrine[10] effects of GM-CSF are mediated via heterologous cell-surface receptors[11] reported to stimulate myeloid cell survival at low GM-CSF concentrations, and survival, proliferation, differentiation, and antimicrobial functions at high concentrations.[12] Normally, GM-CSF is present at very low or undetectable levels in the serum and tissues in both mice and humans.[5,13] Nonetheless, these low levels are critical because GM-CSF–deficient mice have impaired myeloid cell functions, increased mortality from microbial infections, and a lung phenotype characterized by progressive surfactant accumulation as a result of impaired alveolar macrophage surfactant catabolism.[3,5,14-17]

Autoimmune pulmonary alveolar proteinosis (PAP) is a human disease characterized by high levels of GM-CSF autoantibodies and respiratory insufficiency as a result of pulmonary surfactant accumulation[4,18,19] with features similar in nearly every respect to those seen in GM-CSF knockout mice.[3] Disease pathogenesis is thought to be mediated by GM-CSF autoantibodies, which eliminate GM-CSF bioactivity[20] and impair GM-CSF–dependent myeloid cell functions.[5]

Sustained elevation of GM-CSF also seems to be detrimental because transgenic mice nonspecifically overexpressing GM-CSF develop a fatal syndrome of myeloproliferation and inflammation-related tissue destruction.[21] Furthermore, increased local expression of GM-CSF occurs in rheumatoid arthritis in humans, and neutralization of GM-CSF ameliorates disease development in animal models of rheumatoid arthritis[22] and multiple sclerosis,[23] indicating that GM-CSF may be involved in the pathogenesis of inflammatory and autoimmune diseases.[24] These findings strongly suggest that GM-CSF is tightly maintained at very low but critical levels in both humans and mice.

GM-CSF autoantibodies are consistently detected and comprise the major anti-cytokine activity in pharmaceutical intravenous immunoglobulin (IVIG) prepared from pooled serum of healthy subjects.[25] In contrast, GM-CSF autoantibodies have been rarely detected in the serum of healthy persons[25] and, when present, levels were far lower than in patients with PAP.[26] These seemingly paradoxical findings suggest that the pooled serum used to prepare pharmaceutical IVIG may include serum from perons who seem

Submitted May 5, 2008; accepted September 3, 2008. Prepublished online as *Blood* First Edition paper, October 9, 2008; DOI 10.1182/blood-2008-05-155689.

The online version of this article contains a data supplement.

The publication costs of this article were defrayed in part by page charge payment. Therefore, and solely to indicate this fact, this article is hereby marked "advertisement" in accordance with 18 USC section 1734.

healthy but have high levels of serum GM-CSF autoantibodies. This possibility is consistent with the recent report that 31% of people with autoimmune PAP were asymptomatic.[27] Alternatively, GM-CSF autoantibodies may normally be present in healthy people but at low levels and/or in a form not detected in typical immunoassays. Other cytokine autoantibodies have been reported, although their significance remains unclear.[28,29]

We hypothesized that GM-CSF autoantibodies are ubiquitous in humans and function by scavenging and neutralizing free GM-CSF, thereby reducing nonspecific endocrine signaling and myeloid cell priming. Our experiments address the question of why high levels of GM-CSF autoantibody are virtually 100% specific and sensitive for a diagnosis of autoimmune PAP,[4,19,27,30] yet the level of autoantibody does not correlate with the severity of the disease.[31] We tested the hypothesis that GM-CSF autoantibodies rheostatically reduce myeloid cell functions and, above a critical threshold, eliminate GM-CSF signaling altogether. Results provide an estimate of this critical threshold and its association with PAP, help define the therapeutic window for potential future use of GM-CSF autoantibodies to treat inflammatory or autoimmune diseases, and describe a previously unrecognized potential mechanism of innate immune regulation.

Methods

Participants

The institutional review board of the Cincinnati Children's Hospital Medical Center approved this study. All participants or their legal guardians gave written informed consent; minors gave assent in accordance with the Declaration of Helsinki. Volunteers were enrolled into the study as healthy subjects. This group included 57 women and 15 men; mean (± SE) age was 30 plus or minus 0.63 years. All were nonsmokers, were disease-free without a history of major illness, and were symptom-free at the time of enrollment in the study. Patients with autoimmune PAP were recruited from the Rare Lung Diseases Clinic at the University of Cincinnati Medical Center, the Cincinnati Children's Hospital Medical Center, or Niigata Medical and Dental University. The diagnosis of autoimmune PAP was based on clinical and radiographic findings, an open lung biopsy, transbronchial lung biopsy, or cytologic analysis of bronchoalveolar lavage cells and fluid[4] and a positive GM-CSF autoantibody test.[5] This group included 12 women and 11 men; mean age (± SE) was 38 plus or minus 4.0. Of these, 2 (1 man, 1 woman) were in remission (defined as being asymptomatic at the time of enrollment and not requiring treatment of PAP lung disease in the preceding 5 years). All others had active PAP lung disease (defined as having symptoms typical of PAP [eg, dyspnea] and an ongoing requirement for treatment for PAP lung disease at the time of enrollment into the study).

Reagents

Phycoerythrin-conjugated anti–human CD11b antibodies (347557)[5] and fluorescein isothiocyanate–conjugated anti–human CD16 antibodies (555406)[5] were from BD Biosciences (San Jose, CA). Antibodies against STAT5 (SC-835) and actin (SC-1616) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against STAT5A (71-2400) and STAT5B (71-2500) were from Zymed Laboratories (South San Francisco, CA). The phospho-STAT5 antibody (05-495) was from Millipore (Billerica, MA). Horseradish peroxidase (HRP)–conjugated goat anti–human IgG antibodies (A-2290) and rabbit anti–bovine serum albumin (BSA; B-7276)[32] were from Sigma-Aldrich (St Louis, MO). Rabbit anti–human GM-CSF polyclonal antibodies (AB-9667) were from Abcam (Cambridge, MA). Biotinylated goat anti–human GM-CSF polyclonal antibodies (BAF215) were from R&D Systems (Minneapolis, MN). Recombinant human GM-CSF was from Berlex Laboratories (Wayne, NJ; yeast-derived, glycosylated form [Leukine]), Invitrogen (Carlsbad, CA; *Escherichia coli*–derived, unglycosylated form), or PerkinElmer Life and Analytical Sciences (*E coli*–derived, ^{125}I-labeled). The GM-CSF–dependent cell line TF-1 (CRL-2003) was from ATCC. Recombinant IL-8 was from R&D Systems. Protein G columns (17-0404-01) and HiTrap NHS-activated affinity chromatography columns (17-0716-01) were from GE Healthcare (Chalfont St Giles, United Kingdom). Microcon YM-100 filters (42 424) were from Millipore. Nile red–labeled fluorescent microspheres (FP-2056-2) were from Spherotech (Lake Forest, IL). Diethylenetriamine pentaacetic acid (D6518) and ExtrAvidin HRP solution (E2886) were from Sigma-Aldrich. The RIPA Lysis Buffer Kit (24948) was from Santa Cruz Biotechnology.

Purification of GM-CSF autoantibodies

IgG was isolated from serum or commercial IVIG using protein G affinity chromatography as directed by the manufacturer. To remove bound GM-CSF, purified IgG was subjected to ultrafiltration under acidic conditions, pH 2.8, using Microcon YM-100 filters as directed by the manufacturer.[29] IgG recovered from the retentate cup in phosphate-buffered saline (PBS), pH 7.4, was further purified by GM-CSF affinity chromatography on GM-CSF–coupled NHS HiTrap columns as described previously.[5,20] In brief, purified, ultrafiltered IgG was loaded onto the GM-CSF affinity column, and the effluent and 10 mL wash buffer was collected (unbound fraction). GM-CSF–bound proteins were then eluted using 10 mL 100 mmol/L glycine-HCl (pH 2.8; bound fraction).

Far-Western blotting

Bound or unbound immunoglobulin fractions from GM-CSF affinity chromatography were fractionated by sodium dodecyl sulfate (SDS)–polyacrylamide electrophoresis (PAGE) on 2% to 15% gradient gels under nonreducing conditions (30 mA, 150 minutes). Fractionated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by electroblotting (12 volts, 75 minutes). Membranes were incubated with blocking solution (PBS containing 1% (wt/vol) BSA and 0.1% (vol/vol) Tween 20; 4°C, overnight), washed, and then incubated with ^{125}I–GM-CSF (0.16 nM, room temperature, 1 hour), washed, and subjected to autoradiography to localize bound GM-CSF.

Liquid chromatography/tandem mass spectroscopy

GM-CSF–bound proteins were fractionated by gel electrophoresis as above. The single visible bands corresponding in molecular mass to that of IgG were cut out of the gel, minced, digested with trypsin (37°C, overnight), and evaluated with Micromass Quadrupole Time-Of-Flight II mass spectrometer (Waters, Milford, MA). Results were analyzed using the Mascot search engine.[33,34] The top 50 best matching proteins for each subject were analyzed. All matches had a probability-based molecular weight search (Mowse) score over 64 that indicated a specific, nonrandom match (P value $< .05$).

ELISA

IgG subclasses. The concentration of IgG subclasses in affinity-purified GM-CSF autoantibodies was measured using a commercial enzyme-linked immunosorbent assay (ELISA [99-1000; Zymed Laboratories]).

GM-CSF autoantibody. Serum GM-CSF autoantibody levels were measured using a sandwich ELISA as described previously[5,20] with slight modifications. In brief, microtiter plates (96-well, Maxisorp; Nalge Nunc International, Rochester, NY) were coated with recombinant human GM-CSF (1 μg/mL in PBS, 4°C, overnight), washed (PBS containing 0.1% Tween 20), blocked with Stabilcoat (room temperature, 1 hour; Surmodics, Eden Prairie, MN). Serum samples were diluted 1/100 (for healthy subjects) or 1/3000 (for patients with PAP) in sample dilution buffer (PBS, 1% [wt/vol] BSA, 0.1% [vol/vol] Tween 20), and 50 μL were incubated in duplicate wells (room temperature, 40 minutes). Plates were washed and incubated with ammonium acetate (10 mM, pH 5.0, room temperature, 15 minutes) to remove nonspecific binding. Bound IgGs were detected with goat anti–human IgG-HRP diluted 1/3000 with sample dilution buffer