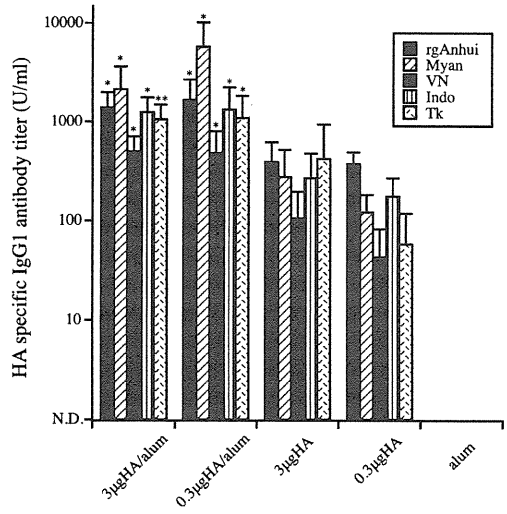

Fig. 6. Viral HA-specific IgG1 antibody titers in sera from vaccinated mice. Mice were vaccinated twice at a 3-week interval and with or without alum. On day 21 after the second vaccination, sera were collected, and viral HA-specific IgG1 antibody titers against various clades of H5N1 viruses were measured by ELISA. Each bar represents the mean value of viral HA-specific IgG1 antibody titers, and error bars represent standard deviations. *$p < 0.05$ for comparison against each vaccine antigen only using Student's *t*-test. **$p < 0.05$ for comparison against each vaccine antigen only using Mann–Whitney *U* test.

antibody-mediated protection against influenza A virus infection [28,29]. Thus, we used ELISA to measure HA-specific IgG1 and IgG2a antibody titers in the sera of vaccinated mice. Sera subjected to ELISA were the same as those used for the titration of virus-neutralizing antibodies.

All vaccinated mice developed HA-specific IgG1 and IgG2a antibodies (Figs. 6 and 7). These antibodies could not be detected in the sera of control mice. Alum significantly enhanced the induction of cross-reactive IgG1 ($p < 0.05$, Student's *t*-test or Mann–Whitney *U* test) and IgG2a ($p < 0.01$, Student's *t*-test or Mann–Whitney *U* test) antibodies against all test HAs from H5N1 viruses belonging to different clades. In particular, IgG1 antibody levels at a low antigen dose were efficiently enhanced by addition of adjuvant, and IgG1 antibody titers at the low antigen dose were as high against all test viral HAs as those at the high antigen dose (Fig. 6). It is noteworthy that IgG1 antibody titers against the HA from the antigenic variant Myan virus were higher, but not significantly so ($p = 0.055$, Student's *t*-test), than those against the HA from rgAnhui vaccine virus in the sera of mice vaccinated with adjuvanted vaccine (Fig. 6), although the virus-neutralizing antibody titer against Myan virus was significantly lower than that against JWE virus ($p < 0.05$, Steel's method for multiple comparison), and was as low as that against clade 1 virus VN (Fig. 1). Similarly, high levels of IgG2a antibodies against Myan and VN viruses were detected by ELISA.

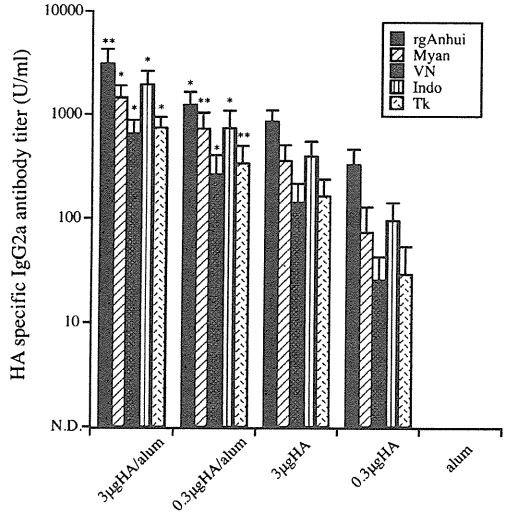

Fig. 7. Viral HA-specific IgG2a antibody titers in sera from vaccinated mice. Mice were vaccinated twice at a 3-week interval and with or without alum. On day 21 after the second vaccination, sera were collected, and viral HA-specific IgG2a antibody titers against various clades of H5N1 viruses were measured by ELISA. Each bar represents the mean value for viral HA-specific IgG2a antibody titers, and error bars represent standard deviation. *$p < 0.01$ for comparison against each vaccine antigen only using Student's *t*-test. **$p < 0.01$ for comparison against each vaccine antigen only using Mann–Whitney *U* test.

4. Discussion

In the present study, we evaluated the vaccine efficacy of rgAnhui virus (clade 2.3.4) with respect to immunogenicity and cross-protection against viruses of homologous and heterologous genetic clades, including antigenic variants. To assess immunogenicity, we detected virus-neutralizing antibody rather than HI antibody, as in our previous mouse model study of H5N1 vaccines, HI antibody was not detected after vaccination at the highest dose (2 μg HA) of adjuvanted vaccine, while sufficient levels of virus-neutralizing antibody were detected in many cases [13]. Furthermore, virus-neutralizing assay is a hallmark for assessing the immunogenicity of H5N1 vaccines due to its higher sensitivity. In the present mouse model experiments, vaccination with 0.3 μg HA enabled the production of virus-neutralizing antibodies against homologous clade 2.3.4 viruses, except for the antigenic variant Myan virus (Fig. 1).

As expected and as reported previously [13,15], the addition of alum to 0.3 μg HA markedly increased antibody levels to levels similar to those induced by vaccination with 3 μg HA antigen. Furthermore, these antibodies were also able to neutralize homologous Myan and heterologous VN strains, and were not observed in mice receiving 3 μg HA antigen without adjuvant. Thus, serum antibodies induced by 0.3 μg HA antigen with alum were able to neutralize all test viruses in clades 1, 2.1.3 and 2.2, as well as homologous clade 2.3.4, including Myan virus. These data clearly indicate that vaccine formulations with alum allow 10-times less vaccine antigen to be used in our mouse model.

Although it has been reported that addition of alum to H5N1 vaccine did not enhance immunogenicity in a clinical study [17], non-adjuvanted H5N1 vaccines required large amounts of vaccine antigen to induce serum antibodies that meet the evaluation criteria for vaccines by the Committee for Proprietary Medicinal Products (CPMP) [12]. The strategy of vaccine antigen sparing is crucial for the creation of a national stockpile of pre-pandemic H5N1 vaccines, as manufacturers have limited vaccine production capacity. Moreover, in our unpublished clinical trials of attenuated and inactivated H5N1 vaccine in 1997, 15 μg HA antigen from split and whole virion vaccines without adjuvant failed to elicit any detectable level of neutralizing antibodies in the sera of vaccine recipients, which led us to the conclusion that an adjuvanted formulation is necessary for H5N1 vaccines. Consequently, the use of alum or other recently approved oil-in-water adjuvants, such as MF59 [17] and AS03 [18], is the most promising and realistic H5N1 vaccine strategy for pandemic preparedness.

In homologous genetic clade 2.3.4, the neutralizing antibody titers induced by rgAnhui vaccine were high against JWE and Laos viruses, but were significantly lower against Myan virus (Fig. 1). Based on antigenic analysis by HI test, JWE and Laos viruses were antigenically related to rgAnhui virus, but the Myan virus differed from the rgAnhui virus (Table 1) through substitution of two amino acids at antigenic site B of the HA protein (data not shown). However, such low cross-reactivity against the Myan virus did not

correlate with the survival rate and weight loss after lethal challenge with Myan virus; all vaccinated mice, regardless of antigen dose and presence or absence of adjuvant, survived without significant weight loss, similarly to mice exposed to other clade 2.3.4 viruses (Figs. 2 and 3).

The protection observed in vaccinated mice should be mediated by virus-specific antibodies, particularly virus-neutralizing and/or viral HA-specific antibodies, as formalin-inactivated viral antigens and alum itself cannot induce cellular immunity [16,30–32]. In fact, on ELISA, the HA-specific IgG1/IgG2a antibodies against Myan virus were markedly elicited with similar levels as homologous vaccine-like virus by non-adjuvanted rgAnhui vaccination (Figs. 6 and 7), while virus-neutralizing antibodies against Myan virus could not be detected (Fig. 1). Moreover, Lu et al. [33] reported that mice vaccinated and survived from following lethal virus challenge did not always possess detectable levels of virus-neutralizing antibodies against challenge virus. It is therefore possible that the undetectable levels of neutralizing antibody induced by vaccination were sufficient to provide protection from the homologous clade of the Myan virus.

Among heterologous clade viruses, virus-neutralizing titers, survival rate and antigenic differences were well correlated. Indo virus (clade 2.1.3) exhibited antigenic similarity to rgAnhui virus on HI test (Table 1) and was well neutralized by antibodies induced by rgAnhui vaccine, similarly to homologous clade 2.3.4 viruses (Fig. 1), and all vaccinated mice survived (Fig. 4B). The results suggest that rgAnhui vaccine sufficiently neutralized Indo-like viruses in clade 2.1.3. On the other hand, rgAnhui vaccination at a low (0.3 μg HA) dose without adjuvant was less effective against clade 1 VN virus, as only 20% of mice challenged with VN virus survived, although the survival rate increased to 80% with addition of adjuvant (Fig. 4A). Such decreased cross-protective efficacy when compared with Indo and Myan viruses, particularly the undetectable levels of virus-neutralizing antibody, may be attributable to the magnitude of antigenic differences between VN and rgAnhui viruses, as HI titers of VN virus were 64-fold lower when compared to the homologous titer of anti-rgAnhui ferret antiserum, and the VN virus apparently differed greatly in antigenicity when compared with the rgAnhui vaccine virus (Table 1). The results indicate that, for the rgAnhui vaccine, enhanced immunogenicity due to addition of adjuvant and/or increasing the antigen dose is necessary to neutralize clade 1 viruses.

The rgAnhui vaccine induced intermediate levels of cross-reactive neutralizing antibody against clade 2.2 Tk virus (Fig. 1), and only a low dose of non-adjuvanted vaccine showed reduced cross-protection (60%) to challenge infection with Tk virus (Fig. 4C). This result was also correlated with the magnitude of antigenic difference between Tk virus and rgAnhui virus, as the HI titer of Tk virus was 16-fold lower when compared with the homologous titer (Table 1). The survival rate of control mice challenged with Tk virus was 40%, in contrast to the 0% seen with other viruses, despite inoculation with the $20 \times MLD_{50}$ dose calculated by the Reed and Muench method. However, 1–2 of the 5 control mice challenged with $20 \times MLD_{50}$, or higher doses, of Tk virus survived in several independent experiments (data not shown). As all control mice challenged with Tk virus showed unrecoverable weight loss (Fig. 5C) and virus-neutralizing antibody titers in the sera of surviving control mice on day 14 after challenge were comparable to those of vaccinated mice (data not shown), the higher survival rate in control mice was not due to a technical failure, but was attributable to the features of the TK virus itself.

Addition of adjuvant improved the protection efficacy against TK virus, but the severity of weight loss in vaccinated mice was markedly higher than with clade 1 VN virus (Fig. 5A and C), despite intermediate levels of neutralizing antibodies being elicited by rgAnhui vaccine. The MLD_{50} values for VN and Tk viruses were 5.1×10^2 $TCID_{50}$ and 2.3×10^6 $TCID_{50}$, respectively. Consequently, the input amount of Tk virus for lethal infection was 4510-times greater when compared to VN virus. This difference may have been reflected in the severity of weight loss.

Despite undetectable levels of virus-neutralizing antibody after non-adjuvanted vaccine, all mice infected with Myan and Indo viruses and 20–80% mice infected with VN virus, respectively, survived (Figs. 1, 2 and 4). Nonetheless, high levels of HA-specific IgG1 and IgG2a antibodies against these viruses were elicited (Figs. 6 and 7), but it is unlikely that non-neutralizing HA-specific antibodies contributed to the survival of virus-infected mice, as HA-specific IgG1 and IgG2a antibodies were also induced in the sera of mice vaccinated but did not survived from following lethal virus challenge. Consequently, the method of virus-neutralizing assay may be less sensitive for evaluating mouse survival after lethal challenge infection.

In the present study, the efficacy of H5N1 vaccine derived from clade 2.3.4, rgAnhui, was assessed based on broad immunogenicity and cross-protection by challenge infection. The rgAnhui vaccine was shown in a mouse model to produce broad-spectrum immunity and to protect against not only homologous infections, but also heterologous clade H5N1 HPAIVs, when the vaccine was conjugated with alum to enhance immunogenicity. Human infections with H5N1 HPAIVs have been continuously reported since 2003, and the antigenic features of the isolates from humans, poultry and wild birds have became variable among the HA genetic clades [34]. Although several H5N1 vaccine candidate viruses chosen from 8 clades (1, 2.1, 2.2, 2,2.1, 2.3.2, 2.3.4, 4 and 7) have been developed and updated [24], it is impossible to prepare stockpile vaccines using all recently available vaccine candidates. The rgAnhui vaccine virus possesses broad cross-immunity, and is therefore a promising candidate for the production of stockpile H5N1 vaccine. In fact, rgAnhui virus was selected for the Japanese national stockpile vaccine in 2007 [35].

Acknowledgements

We would like to thank Dr. Phengta Vongprachanh, National Center for Laboratory and Epidemiology, Lao PDR, Dr. Khin Yi Oo, National Health Laboratory, Myanmar, and Dr. L.T.Q. Mai, National Institute of Health and Epidemiology, Viet Nam, for providing clinical specimens for isolation of A/Laos/JP127/2009, A/Myanmar/JPA007-07/2007 and A/VietNam/JP1203/2004, respectively. We are also grateful to Dr. Nancy Cox, US-CDC, Dr. Alan Hay, National Institute of Medical Research, and Dr. Malik Peiris, University of Hong Kong, for providing rgAnhui01/2005 vaccine virus and post-infection ferret sera, A/Turkey/12/2006, A/Indonesia/5/2005 and A/Japanese White Eye/HK/1038/2005, respectively. These studies were supported by a Health Labor Sciences Research Grant from the Ministry of Health, Labor and Welfare of Japan and by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

References

[1] Claas EC, Osterhaus AD, van Beek R, De Jong JC, Rimmelzwaan GF, Senne DA, et al. Human influenza A H5N1 virus related to a highly pathogenic avian influenza virus. Lancet 1998;351(February (9101)):472–7.
[2] Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, et al. Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science 1998;279(January (5349)):393–6.
[3] World Health Organization. Cumulative number of confirmed human cases of avian influenza A/(H5N1) reported to WHO; 2011, cited, Available from: http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_08_02/en/index.html.
[4] Normile D. Avian influenza human transmission but no pandemic in Indonesia. Science 2006;312(June (5782)):1855.

[5] Tran TH, Nguyen TL, Nguyen TD, Luong TS, Pham PM, Nguyen VC, et al. Avian influenza A (H5N1) in 10 patients in Vietnam. N Engl J Med 2004;350(March (12)):1179–88.
[6] Ungchusak K, Auewarakul P, Dowell SF, Kitphati R, Auwanit W, Puthavathana P, et al. Probable person-to-person transmission of avian influenza A (H5N1). N Engl J Med 2005;352(January (4)):333–40.
[7] Ministry of Agriculture FaFoJ; 2011, cited; Available from: http://www.maff.go.jp/j/syouan/douei/tori/pdf/110405_ai_japan_map.pdf.
[8] Ministry of Health LaW; 2004, cited; Available from: http://www.mhlw.go.jp/topics/bukyoku/kenkou/tori/041222/1.html.
[9] Nicolson C, Major D, Wood JM, Robertson JS. Generation of influenza vaccine viruses on Vero cells by reverse genetics: an H5N1 candidate vaccine strain produced under a quality system. Vaccine 2005;23(April (22)):2943–52.
[10] Dong J, Matsuoka Y, Maines TR, Swayne DE, O'Neill E, Davis CT, et al. Development of a new candidate H5N1 avian influenza virus for pre-pandemic vaccine production. Influenza Other Respi Viruses 2009;3(November (6)):287–95.
[11] Webby RJ, Perez DR, Coleman JS, Guan Y, Knight JH, Govorkova EA, et al. Responsiveness to a pandemic alert: use of reverse genetics for rapid development of influenza vaccines. Lancet 2004;363(April (9415)):1099–103.
[12] Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med 2006;354(March (13)):1343–51.
[13] Ninomiya A, Imai M, Tashiro M, Odagiri T. Inactivated influenza H5N1 whole-virus vaccine with aluminum adjuvant induces homologous and heterologous protective immunities against lethal challenge with highly pathogenic H5N1 avian influenza viruses in a mouse model. Vaccine 2007;25(May (18)):3554–60.
[14] Brady RC, Treanor JJ, Atmar RL, Keitel WA, Edelman R, Chen WH, et al. Safety and immunogenicity of a subvirion inactivated influenza A/H5N1 vaccine with or without aluminum hydroxide among healthy elderly adults. Vaccine 2009;27(August (37)):5091–5.
[15] Lin J, Zhang J, Dong X, Fang H, Chen J, Su N, et al. Safety and immunogenicity of an inactivated adjuvanted whole-virion influenza A (H5N1) vaccine: a phase I randomised controlled trial. Lancet 2006;368(September (9540)):991–7.
[16] Naim JO, van Oss CJ, Wu W, Giese RF, Nickerson PA. Mechanisms of adjuvancy: I—metal oxides as adjuvants. Vaccine 1997;15(August (11)):1183–93.
[17] Bernstein DI, Edwards KM, Dekker CL, Belshe R, Talbot HK, Graham IL, et al. Effects of adjuvants on the safety and immunogenicity of an avian influenza H5N1 vaccine in adults. J Infect Dis 2008;197(March (5)):667–75.
[18] Leroux-Roels G. Prepandemic H5N1 influenza vaccine adjuvanted with AS03: a review of the pre-clinical and clinical data. Expert Opin Biol Ther 2009;9(August (8)):1057–71.
[19] Europian Medicines Agency. Aflunov, cited; Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002094/human_med_001396.jsp&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058001d125.
[20] Europian Medicines Agency. Prepandemic influenza vaccine (H5N1) Novartis Vaccines and Diagnostic, cited; Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002269/human_med_001397.jsp&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058001d125.
[21] Europian Medicines Agency. Prepandrix, cited; Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000822/human_med_000986.jsp&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058001d125.
[22] Europian Medicines Agency. Pandemic influenza vaccine (H5N1) (split virion, inactivated, adjuvanted) GlaxoSmithKline Biologicals, cited; Available from: http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/001206/human_med_001214.jsp&murl=menus/medicines/medicines.jsp&mid=WC0b01ac058001d125.
[23] World Health Organization. Antigenic and genetic characteristics of H5N1 viruses and candidate H5N1 vaccine viruses developed for potential use as pre-pandemic vaccines; 2005, cited; Available from: http://www.who.int/csr/disease/avian_influenza/guidelines/recommendationvaccine.pdf.
[24] World Health Organization. Antigenic and genetic characteristics of influenza A (H5N1) and influenza A(H9N2) viruses and candidate vaccine viruses developed for potential use in human vaccines; 2011, cited; Available from: http://www.who.int/csr/disease/avian_influenza/guidelines/2011_02_h5_h9_vaccinevirusupdate.pdf.
[25] Takahashi Y, Hasegawa H, Hara Y, Ato M, Ninomiya A, Takagi H, et al. Protective immunity afforded by inactivated H5N1 (NIBRG-14) vaccine requires antibodies against both hemagglutinin and neuraminidase in mice. J Infect Dis 2009;199(June (11)):1629–37.
[26] Brewer JM, Conacher M, Hunter CA, Mohrs M, Brombacher F, Alexander J. Aluminium hydroxide adjuvant initiates strong antigen-specific Th2 responses in the absence of IL-4- or IL-13-mediated signaling. J Immunol 1999;163(December (12)):6448–54.
[27] Brewer JM, Conacher M, Satoskar A, Bluethmann H, Alexander J. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to freund's complete adjuvant, but continues to induce T helper 2 cytokine production. Eur J Immunol 1996;26(September (9)):2062–6.
[28] Fazekas G, Rosenwirth B, Dukor P, Gergely J, Rajnavolgyi E. IgG isotype distribution of local and systemic immune responses induced by influenza virus infection. Eur J Immunol 1994;24(December (12)):3063–7.
[29] Palladino G, Mozdzanowska K, Washko G, Gerhard W. Virus-neutralizing antibodies of immunoglobulin G (IgG) but not of IgM or IgA isotypes can cure influenza virus pneumonia in SCID mice. J Virol 1995;69(April (4)):2075–81.
[30] Bomford R. The comparative selectivity of adjuvants for humoral and cell-mediated immunity. II. Effect on delayed-type hypersensitivity in the mouse and guinea pig, and cell-mediated immunity to tumour antigens in the mouse of Freund's incomplete and complete adjuvants, alhydrogel, Corynebacterium parvum, Bordetella pertussis, muramyl dipeptide and saponin. Clin Exp Immunol 1980;39(February (2)):435–41.
[31] Reiss CS, Schulman JL. Cellular immune responses of mice to influenza virus vaccines. J Immunol 1980;125(November (5)):2182–8.
[32] Wijburg OL, van den Dobbelsteen GP, Vadolas J, Sanders A, Strugnell RA, van Rooijen N. The role of macrophages in the induction and regulation of immunity elicited by exogenous antigens. Eur J Immunol 1998;28(February (2)):479–87.
[33] Lu X, Edwards LE, Desheva JA, Nguyen DC, Rekstin A, Stephenson I, et al. Cross-protective immunity in mice induced by live-attenuated or inactivated vaccines against highly pathogenic influenza A (H5N1) viruses. Vaccine 2006;24(November (44–46)):6588–93.
[34] World Health Organization. Weekly epidemiological record; 2011, cited; Available from: http://www.who.int/wer/2011/wer8617.pdf.
[35] Ministry of Health LaW. Annual health, labour and welfare report 2007–2008; 2008, cited; Available from: http://www.mhlw.go.jp/english/wp/wp-hw2//part2//p2c1s7a.pdf.

International Immunology, Vol. 23, No. 7, pp. 433–441
doi:10.1093/intimm/dxr031
Advance Access publication 3 June 2011

HIV-1 Nef impairs multiple T-cell functions in antigen-specific immune response in mice

Hideki Fujii[1,2,*], Manabu Ato[2,*], Yoshimasa Takahashi[2], Kaori Otake[3], Shu-ichi Hashimoto[4], Tomohiro Kaji[5], Yasuko Tsunetsugu-Yokota[2], Mikako Fujita[6], Akio Adachi[7], Toshinori Nakayama[8], Masaru Taniguchi[9], Shigeo Koyasu[1] and **Toshitada Takemori[2,5]**

[1]Department of Microbiology and Immunology, Keio University School of, Medicine, Tokyo 160-8582, Japan
[2]Department of Immunology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan
[3]Gifu Social Insurance Hospital, Gifu 509-0206, Japan
[4]Chiome Bioscience Inc., Saitama 351-0104, Japan
[5]Laboratory for Immunological Memory, Riken Research Center for Allergy and Immunology, Kanagawa 230-0045, Japan
[6]Department of Bioorganic Medicinal Chemistry, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, Kumamoto 862-0973, Japan
[7]Department of Virology, Institute of Health Biosciences, The University of Tokushima Graduate School, Tokushima 770-8503, Japan
[8]Department of Immunology, Graduate School of Medicine, Chiba University, Chiba 260-8670, Japan
[9]Laboratory for Immune Regulation, Riken Research Center for Allergy and Immunology, Kanagawa 230-0045, Japan

*These authors contributed equally to this study.

Correspondence to: T. Takemori; E-mail: mttoshi@rcai.riken.jp

Received 10 February 2011, *accepted* 2 May 2011

Abstract

The viral protein Nef is a key element for the progression of HIV disease. Previous *in vitro* studies suggested that Nef expression in T-cell lines enhanced TCR signaling pathways upon stimulation with TCR cross-linking, leading to the proposal that Nef lowers the threshold of T-cell activation, thus increasing susceptibility to viral replication in immune response. Likewise, the *in vivo* effects of Nef transgenic mouse models supported T-cell hyperresponse by Nef. However, the interpretation is complicated by Nef expression early in the development of T cells in these animal models. Here, we analyzed the consequence of Nef expression in ovalbumin-specific/CD4$^+$ peripheral T cells by using a novel mouse model and demonstrate that Nef inhibits antigen-specific T-cell proliferation and multiple functions required for immune response *in vivo*, which includes T-cell helper activity for the primary and memory B-cell response. However, Nef does not completely abrogate T-cell activity, as defined by low levels of cytokine production, which may afford the virus a replicative advantage. These results support a model, in which Nef expression does not cause T-cell hyperresponse in immune reaction, but instead reduces the T-cell activity, that may contribute to a low level of virus spread without viral cytopathic effects.

Keywords: AIDS, acquired immunity, humoral response

Introduction

The Nef protein of the primate lentiviruses HIV-1/2 and the simian immunodeficiency virus (SIV) is expressed from the earliest stage of viral gene expression (reviewed in ref. 1). Nef-defective viruses cause a slow progression of clinical disease with reduced viral loads in humans and rhesus macaques with HIV-1/2 and SIV infection, respectively, indicating that Nef plays a crucial role in viral pathogenesis in human and non-human primates (reviewed in ref. 1). Nef associates with host cell membranes through N-terminal myristoylation and functions as an adaptor bringing together a large number of proteins in host cells, mainly protein kinases and components of endocytic trafficking machinery (reviewed in ref. 1; refs 2–7).

Nef reduces surface level receptors, including CD4, the primary receptor for HIV and SIV and MHC class I and class II complex, facilitating HIV immune evasion and thus increases viral pathogenesis (reviewed in ref. 1). Additionally, extensive *in vitro* studies, mostly carried out by using human T-cell lines, have suggested that Nef expression enhances TCR-mediated signaling pathways and transcriptional activation (reviewed in ref. 1; refs 2–5). Such alterations in signaling events may lower the TCR activation threshold in CD4$^+$

T cells and help more responsive to T-cell activation signals, a process that could support higher virus production upon stimuli mediated via the TCR (reviewed in ref. 1; refs 2–5). Moreover, Nef may alter host cell death pathways to prevent apoptosis of infected cells, thereby fostering their longevity (reviewed in ref. 1) These observations have led to a model in which Nef reorganizes the host cell activity so as to optimize viral propagation and cell survival, thus facilitating immune evasion and participating in virus spread.

The consequence of Nef expression in primary cells has been examined by using Nef transgenic (Tg) mice, in which Nef was constitutively or transiently expressed under control of a T-cell-specific promoter–enhancer element (8, 9). In this model system, Nef promotes T-cell activation, however, interpretation of these findings is complicated by the fact that expression of Nef early in the development of T cells results in wholesale depletion of thymocytes and peripheral T cells. Moreover, it remains obscure whether the T-cell activation seen in Nef Tg mice is mediated by lymphopenia-induced mechanisms rather than by an intrinsic effect of Nef expression on T-cell activation and proliferation (9, 10).

In the present study, to examine the consequence of Nef expression in primary cells, we established a double transgenic mouse (dTg), which expresses human coxsackie/adenovirus receptor (CAR) (11) and an ovalbumin (OVA)-specific TCR that recognizes the OVA peptide on antigen-presenting cell (APC) with high affinity under MHC Class II I-A^d-restriction. This system allowed us to analyze the effect of Nef on antigen-specific peripheral T-cell function by transfer of the *nef* gene into peripheral T cells using an adenovirus vector. The present study demonstrates that Nef expression does not cause T-cell hyperresponse but instead impairs T-cell functions required for immune response.

Methods

Mice

BALB/c and CB17-scid mice were purchased from Shizuoka Laboratory Animal Center (Hamamatsu, Japan) and Clea Japan, Inc. (Tokyo, Japan), respectively. Tg mice expressing the CAR under the control of the Lck proximal promoter (CAR Tg mice) on the BALB/c background have been described previously (11). DO11.10 mice express a transgenic TCR with specificity for OVA peptide residues 323–339 ($OVA_{323–339}$) restricted by I-A^d on the BALB/c background (12). All mice used in this study were maintained under specific pathogen-free conditions and used at 6–12 weeks of age in accordance with the guidelines of the Institutional Animal Care and Use Committee, National Institute of Infectious Diseases.

Adenovirus vector

Recombinant adenovirus vectors were generated using the AdEasy Adenoviral Vector System (Stratagene) according to the manufacturer's instructions. In order to express the *nef* gene under the CAG promoter, the pShuttle vector was digested with *Kpn*I, blunt-ended with T4 polymerase and then, the CAG promoter DNA was ligated (pShuttle-CAG). Next, an *Xho*I–*Xba*I fragment of pIRES2-EGFP (Invitrogen) was inserted into the *Xho*I–*Xba*I site of pShuttle-CAG, which was designated as pShuttle-CAG-I2-EGFP. HIV-1 NL4-3 nef wild-type and a mutant ($^{57}W^{58}L$ to $^{57}A^{58}A$) were PCR amplified from pNL432 and pNL-n57/2A proviral DNA, respectively, using specific primers containing *Eco*RI sites at both ends and then subcloned into pBluscript KS^+ (Stratagene). The *Eco*RI fragment containing wild-type or mutant nef was inserted into the *Eco*RI site of pShuttle-CAG-I2-EGFP. These shuttle vectors were linearized and co-transformed into *Escherichia coli* strain BJ5183-AD-1, which contains the pAdEasy vector, to induce homologous recombination (Supplementary Figure 1 is available at *International Immunology* Online). Recombinant adenoviral plasmids were selected and transfected into 293 cells to produce recombinant adenovirus particles. Recombinant adenovirus were purified by two rounds of Cesium chloride density gradient centrifugation as described previously (13). The concentrated virus was dialyzed against PBS containing 10% glycerol. The titer of the virus stock was determined by a plaque formation assay using 293 cells.

T-cell purification and recombinant adenovirus infection

For recombinant adenovirus infection, $CD4^+$ T cells were enriched by negative selection on a MACS column (Miltenyi Biotec GmbH, Gladbach, Germany) as previously described (14). Briefly, cells were blocked with anti-FcγRII/III (2.4G2; BD PharMingen, San Diego, CA, USA) and incubated with biotinylated mAbs against B220(RA3-6B2), IgM(II/41), IgD(11-26), Gr1(RB6-8C5), CD11c(N418), CD49b(DX5), CD11b(M1/70) and CD8(53–6.7) (eBioscience, San Diego, CA, USA), followed by incubation with streptavidin-coated microbeads (Miltenyi Biotec GmbH). Purified $CD4^+$ T cells (>95%) were infected with recombinant adenovirus vector at a multiplicity of infection of 10 (MOI 10) for 2 days in 24-well plates at a concentration of 2×10^6 per well in RPMI 1640 medium supplemented with 10% Fetal Bovine Serum (FBS), 5×10^5 M 2-mercaptoethanol, L-glutamine, antibiotics and IL-7 (20 ng ml^{-1}; PeproTech, London, UK) at 37°C in an atmosphere of 5% CO_2.

Proliferation assays and ELISA

Sorted $CD4^+$ GFP^+ T cells were cultured in microtiter wells at a concentration of 4×10^4 cells per well in the presence of $OVA_{323–339}$ peptide and 5×10^5 irradiated T-depleted spleen cells. DNA synthesis of cultured cells in triplicate was estimated by the incorporation of [^{3}H] thymidine (0.5 μCi) added 12 h prior to cell harvest. The level of IFN-γ and IL-2 in the culture supernatants was measured by a Ready-Set-Go! ELISA assay kit (eBioscience), according to the manufacturer's instruction. In some experiments, $CD4^+$ GFP^+ T cells (2×10^6) were cultured for 2–3 days in 96-well plates immobilized with anti-TCR mAb (5 μg ml^{-1}) and anti-CD28 mAb (1 μg ml^{-1}) (BD PharMingen).

Chemotaxis assay

Chemotaxis assays were performed in Transwell (Corning Coster, Coring, NY, USA) with polycarbonate filters (5 μm pore size) as described previously (15). Briefly, purified $CD4^+$ GFP^+ T cells were suspended at 5×10^6 cells ml^{-1} in RPMI 1640 medium containing 1% FBS and 25 mM HEPES. One

hundred microliters of cell suspension was loaded onto the upper wells and placed in a 24-well plate containing 600 μl of media with the indicated doses of CXCL12 (SDF-1α) (PeproTech) or CCL19 (ELC) (R&D Systems, Minneapolis, MN, USA). Cells were incubated at 37°C for 90 min, and cells in the bottom wells were counted using a FACSCalibur.

Activation-induced cell death assay

Sorted $CD4^+$ GFP^+ T cells were cultured at a concentration of 1×10^6 cells ml^{-1} in 96-well plates immobilized with 5 μg ml^{-1} of anti-CD3ε mAb (2C11) (BioLegend, San Diego, CA, USA) in RPMI medium supplemented with 10% FBS. Cells were harvested 2 days later and then re-cultured for 3 days in 96-well plates containing immobilized with anti-CD3 mAb or medium containing 200 U ml^{-1} of human IL-2 (PeproTech). To detect apoptotic cells, a terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay was performed using the ApopTag Red In Situ Apoptosis Detection Kit (CHEMICON International Inc., Temecula, CA, USA). Briefly, the cells were collected and deposited on glass slides by cytospin (Shandon, London, UK), fixed with PBS containing 1% PFA for 10 min and the DNA free 3′ OH were enzymatically labeled with digoxigenin-labeled nucleotides, which were detected using rhodamine-labeled anti-digoxigenin polyclonal antibodies according to the manufacturer's instructions. After applying 6 μg ml^{-1} of Hoechst33342 (Invitrogen) for nuclear staining, slides were processed for analysis using an LSM 510 laser-scanning confocal microscope (Carl Zeiss, Jena, Germany). The proportion of apoptotic cells was determined by counting at least 100 cells in the captured images.

T-cell migration in vivo

BALB/c mice were intravenously injected with 2×10^6 of purified $CD4^+$ GFP^+ T cells uninfected or infected with a recombinant adenovirus vector. Twenty-four hours later, the recipient mice were subcutaneously immunized with 0.2 mg of LPS-free OVA in CFA on the back at three sites. The number of $CD4^+KJ1\text{-}26^+$ T cells in the draining lymph nodes was measured by flow cytometry at 5 days after immunization.

Adoptive cell transfer

Transfer of B cells and OVA-specific/$CD4^+$ T cells infected with a recombinant adenovirus vector in adoptive hosts was performed as described previously (14).

Briefly, $CD4^+$ GFP^+ T cells were prepared by FACS sorting from dTg T cells infected with a recombinant adenovirus vector *in vitro*. B cells were negatively selected from the pooled spleens of either naive mice or 4-hydroxyl-3-nitrophenylacetyl-conjugated chicken γ-globulin (NP-CGG)-primed mice using a MACS system and biotinylated anti-CD5 (53–7.3), anti-CD90.2 (53–2.1), anti-Gr1, anti-CD11b (eBioscience), anti-CD43 (57) and anti-CD138 (281-2) (BD PharMingen). The procedure consistently yielded >95% $B220^+$ cells. Purified B cells (5×10^6) together with $CD4^+$ GFP^+ T cells infected with recombinant adenovirus vector (3×10^4) were intravenously injected into CB17-scid mice. One day later, the recipient mice were intraperitoneally challenged with 25 μg of soluble NP-OVA, and the sera were collected from individual mice at day 7 after challenge. Anti-NP serum antibody titers were estimated by ELISA assays using NP_2-BSA and NP_{18}-BSA as coating antigens as described previously (14). The relative affinity of anti-NP antibodies was estimated by calculating the ratio of anti-NP_2/anti-NP_{18} antibody.

Statistics

The results were evaluated statistically by two-tailed Student's *t*-test ($n = 3$) or Mann–Whitney nonparametric test ($n > 4$), with $P < 0.05$ regarded as significant.

Results

Nef impairs T-cell proliferation upon antigen stimulation in vitro

In order to determine the effect of Nef expression in peripheral T cells, we crossed Tg mice that express an OVA-specific T-cell receptor (12) with mice expressing CAR on T cells (11). OVA-specific/$CD4^+$/CAR^+ T cells were purified from the pooled spleens of dTg mice and infected *in vitro* with an adenovirus vector encoding green fluorescence protein (GFP) driven by the CAG promoter with (Ad-nef) or its mutants [Ad-nef (mu)] or without the *nef* gene (Ad) in the presence of IL-7, which supports T-cell survival and promotes progression into the G_{1b} stage of the cell cycle (16, 17). Thereafter, GFP^+ cells were purified by FACS and provided for analysis as below.

Consistent with previous observations in human T-cell lines, Fig. 1(A) shows that CD4 expression on murine peripheral T cells was down-regulated by Nef but not by the Nef mutant carrying amino acids replacements of $^{57}W^{58}L$ to $^{57}A^{58}A$, abrogating the ability to down-regulate CD4 (18). Nef expression had no effect on the expression of CD25, CD28, CD44, CD62L, CD69, TCRβ and MHC class I (data not shown).

To examine the effect of Nef in T-cell response, GFP^+ cells were purified by FACS from $CD4^+$/CAR^+ T cells infected with Ad-nef, Ad-nef (mu) and cultured in the presence of irradiated splenocytes as APCs, which had been pulsed with OVA peptide ($OVA_{323–339}$). Expression of wild-type as well as mutant forms of Nef diminished T-cell proliferation upon stimulations with OVA peptide at a dose of 0.1 μM (Fig. 1B). These Nef proteins also reduced the level of cytokines produced by T cells in response to different doses of OVA peptide (Fig. 1C). These results suggest that Nef prominently affects T-cell proliferation, irrespective of Nef's ability to down-modulate CD4 but not completely abrogate T-cell activation.

Nef-expression diminishes T-cell migration activity in the primary immune response

Chemokines and their receptors play pivotal roles in the initial homing of lymphocytes and their subsequent trafficking during an immune response (6). It has been reported that Nef impairs the migratory capacity of human T-cell lines *in vitro* in response to the chemokine CXCL12, which binds to T-cell receptor, CXCR4, owing to alteration of the signal cascades downstream of chemokine receptors (7, 15). Consistently, the expression of Nef or its mutant in murine $CD4^+$ T cells reduced their migration in response to CXCL12 *in vitro*, without altering the surface receptor expressions (Fig. 2A and B).

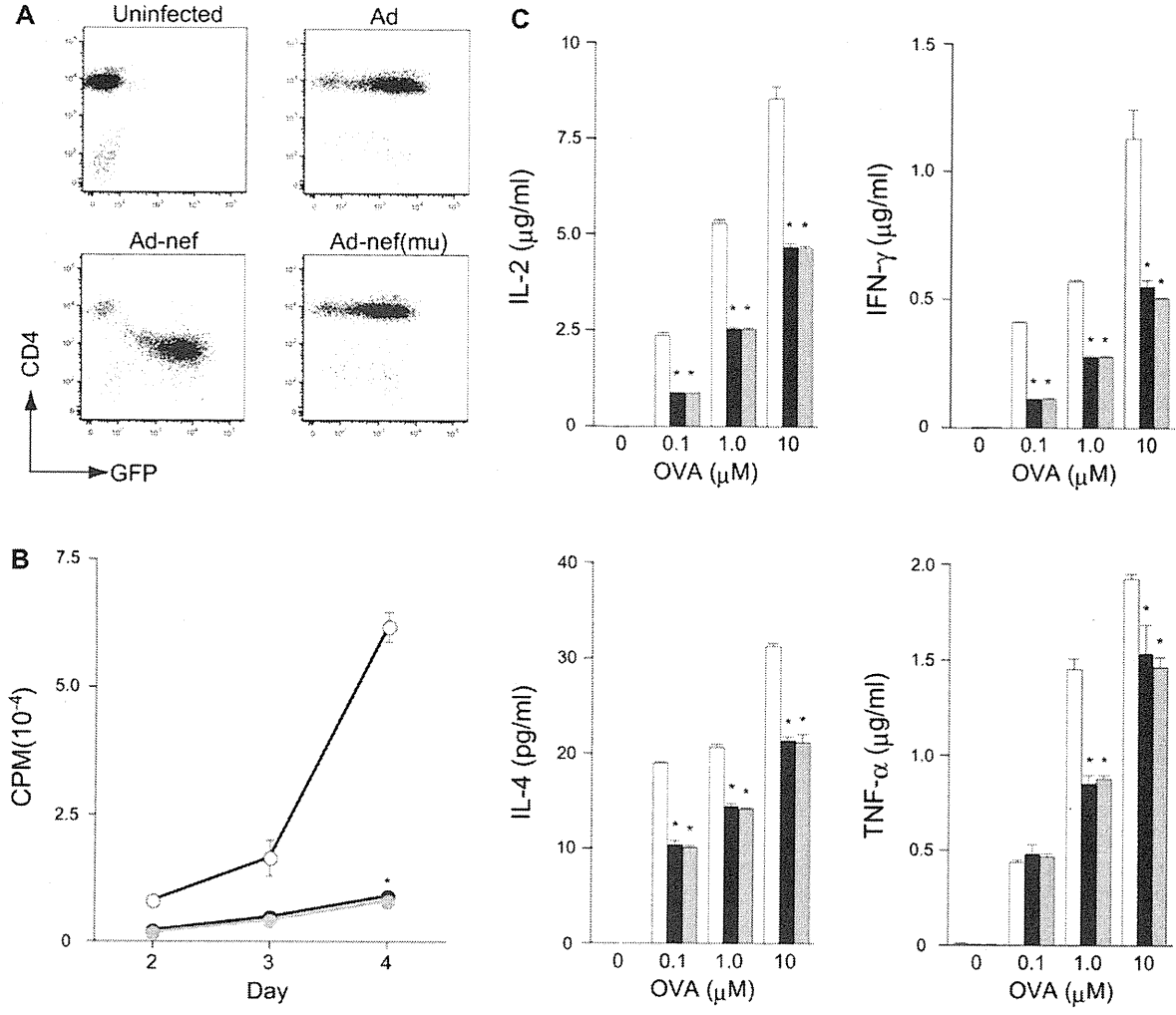

Fig. 1. (A) Characterization of Nef-expressing T cells. A *EGFP* gene-containing adenoviral vector was used to evaluate the efficiency of adenovirus (Ad) infection in DO11.10/CAR$^+$/CD4$^+$ T cells. Naive CD4$^+$ T cells from dTg mice were infected with Ad-nef, Ad-nef (mu) or Ad vector as a control. Two days later, GFP and CD4 expression were assessed by FACS. (B) Nef represses antigen-specific T-cell proliferation. Purified CD4$^+$/GFP$^+$ T cells (5×10^4) infected with Ad-nef (closed), Ad-nef (mu) (gray) and Ad (open) were cultured with T-cell depleted spleen cells as APCs (5×10^5) pulsed with 0.1 μM of $OVA_{323-339}$ peptide. Their DNA synthesis in the triplicate culture was estimated at the indicated periods by the incorporation of [^{3}H] thymidine added 12 h prior to cell harvest. *$P < 0.001$ versus Ad. (C) Purified CD4$^+$ GFP$^+$ T cells and APCs were co-cultured with various concentrations of $OVA_{323-339}$ peptide. Cytokine production in culture supernatant was measured by ELISA on day 3 of culture. *$P < 0.001$ versus Ad. Shown is the representative data from two independent experiments.

Likewise, the Nef proteins, including NL4-3 Nef, did not alter the expression of CXCR4 on human T cells (15, 19), however, there are controversial reports that HIV-1 Nef caused a modest decrease in expression of CXCR4 on human T cells, irrespective of Nef alleles, including NA7 and NL4-3 (7, 20). Further analysis is needed to resolve the discrepancy among these studies.

To examine whether Nef affects T-cell migration *in vivo*, OVA-specific CD4$^+$ T cells were purified from pooled splenocytes of dTg mice and infected with Ad-nef, Ad-nef(mu) or Ad. These cells were transferred into syngeneic recipients, followed by subcutaneous inoculation with OVA in CFA. Five days later, the frequency of OVA-specific (KJ1-26$^+$) CD4$^+$ T cells in the draining lymph node was estimated by FACS. As shown in Fig. 2(C), we observed that Nef impairs the physiological recruitment of T cells into the secondary lymphoid tissues in the immune response. A substantial number of GFP$^+$/OVA-specific/CD4$^+$ T cells infected with Ad accumulated in the draining lymph node after OVA stimulation, however, the number of cells was significantly reduced when the T cells expressed Nef or its mutant. T cells in the draining lymph nodes uniformly expressed high levels of CD44, a marker for activated T cells (21), irrespective of their expression of Nef or Nef mutant (Fig. 2D), suggesting that they were activated, but not involved in functional maturation. These results suggest that Nef affects trafficking of T cells to the regional lymph nodes during an immune response, independently of CD4 down-modulation.

As shown in Fig. 2(E), we examined the possibility that nef expression causes T cells to undergo AICD, which could reduce the number of cells migrating to the regional lymph nodes after stimulation. OVA-specific/CD4$^+$ or

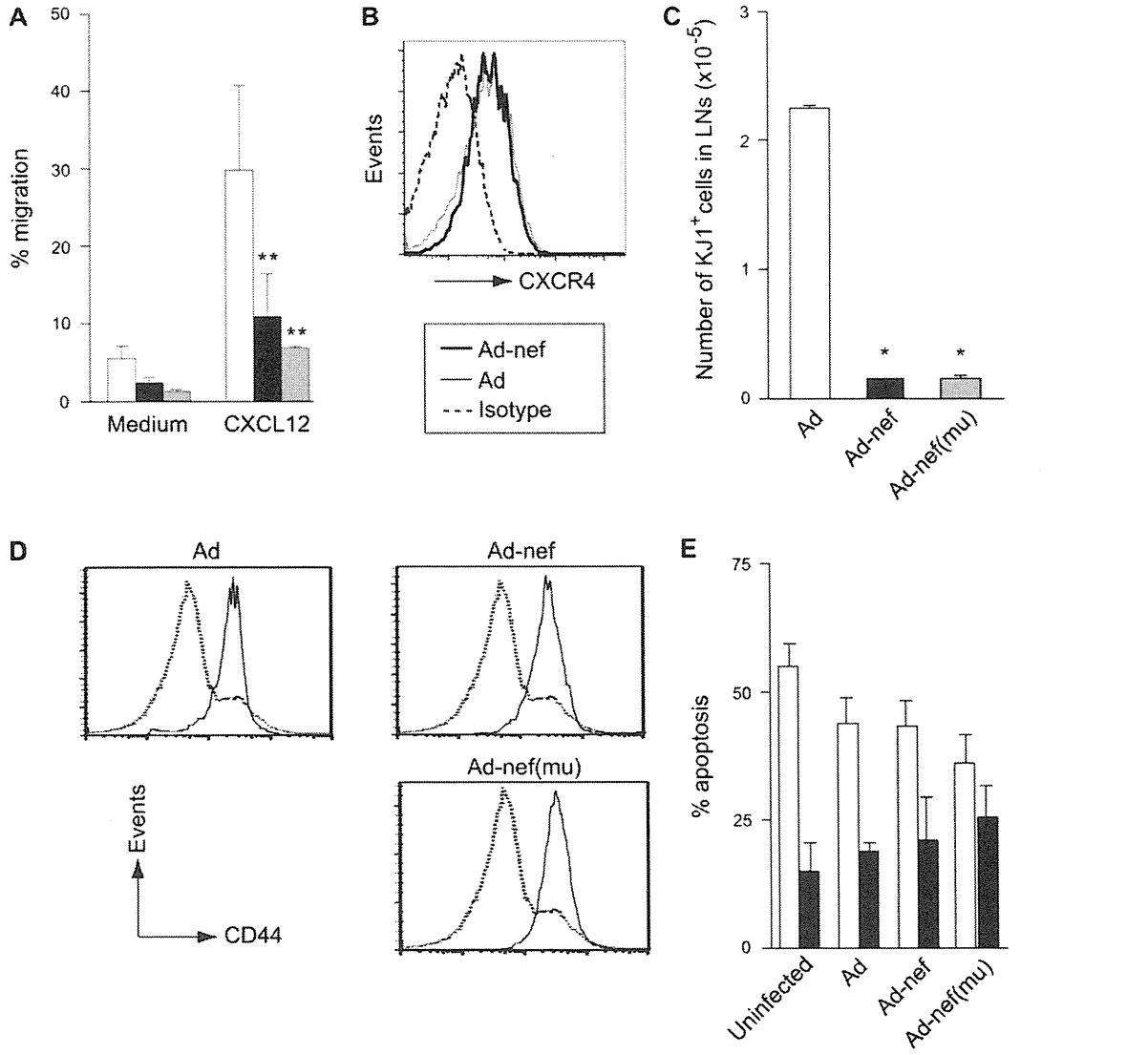

Fig. 2. Nef impairs T-cell migratory activity (A). $CD4^+$ GFP^+ T cells infected with Ad-nef (closed column), Ad-nef (mu) (gray column) and Ad (open column) were used in transwell chemotaxis assays in the presence of CXCL12 (PeproTech). Cells were allowed to migrate in the bottom wells for 90 min, and the proportion of cells that had migrated into the lower wells was determined by flow cytometry. The results are shown as mean ± SD ($n = 3$). *$P < 0.01$ versus Ad. (B) CXCR4 surface staining for $CD4^+$/GFP^+ T cells after infection with Ad-nef (solid line) or Ad (thin line), together with control IgG staining (broken lines). (C and D) $CD4^+$/GFP^+ T cells (2×10^6) infected with Ad-nef (closed column), Ad-nef (mu) (gray column) and Ad (open column) were transferred into BALB/c mice and 24 h later mice were injected subcutaneously with 0.2 mg of LPS-free OVA with CFA on the back in three sites. The cell number (±SD) of $CD4^+$/OVA-specific T cells in the draining lymph nodes (C) and the level of CD44 expression in Ad-infected donor (solid line) and recipient $CD4^+$ T cells (broken line) (D) were measured by flow cytometry using anti-CD4, anti-CD44 and KJ1-26 mAbs on day 5 after OVA injection. *$P < 0.001$ versus Ad. (E) $CD4^+$ T cells (1×10^6) or $CD4^+$/GFP^+ T cells (1×10^6) infected with Ad-nef, Ad-nef (mu) and Ad were stimulated with immobilized anti-CD3ε mAb for 2 days, followed by re-stimulation with anti-CD3 mAb/IL-2 (open column) or IL-2 alone (closed column) for 3 days. Apoptotic cells were analyzed by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labeling assay. Representative data from two independent experiments in (A), (C) and (D) and from three independent experiments (B) is shown.

OVA-specific/$CD4^+$/GFP^+ T cells were hyperstimulated with immobilized anti-CD3ε mAb at 2-day intervals as previously described (22). The results show that Nef did not enhance the induction of AICD in T cells upon TCR-stimulation *in vitro* nor did it compromise the survival function mediated by IL-2. Therefore, it seems unlikely that Nef causes T-cell death, which could reduce the number of cells migrating to the regional lymph nodes.

Nef expression in T cells affects the primary and memory B-cell responses

To examine T-cell helper activity by Nef, OVA-specific/$CD4^+$ T cells were purified from the pooled spleens of dTg mice, followed by infection with or without Ad-nef, Ad-Nef (mu) or Ad. The GFP^+/$CD4^+$ T cells were purified by FACS (Fig. 3A) and transferred into CB17-scid mice, together with either naive or NP-primed B cells. The recipients were immunized

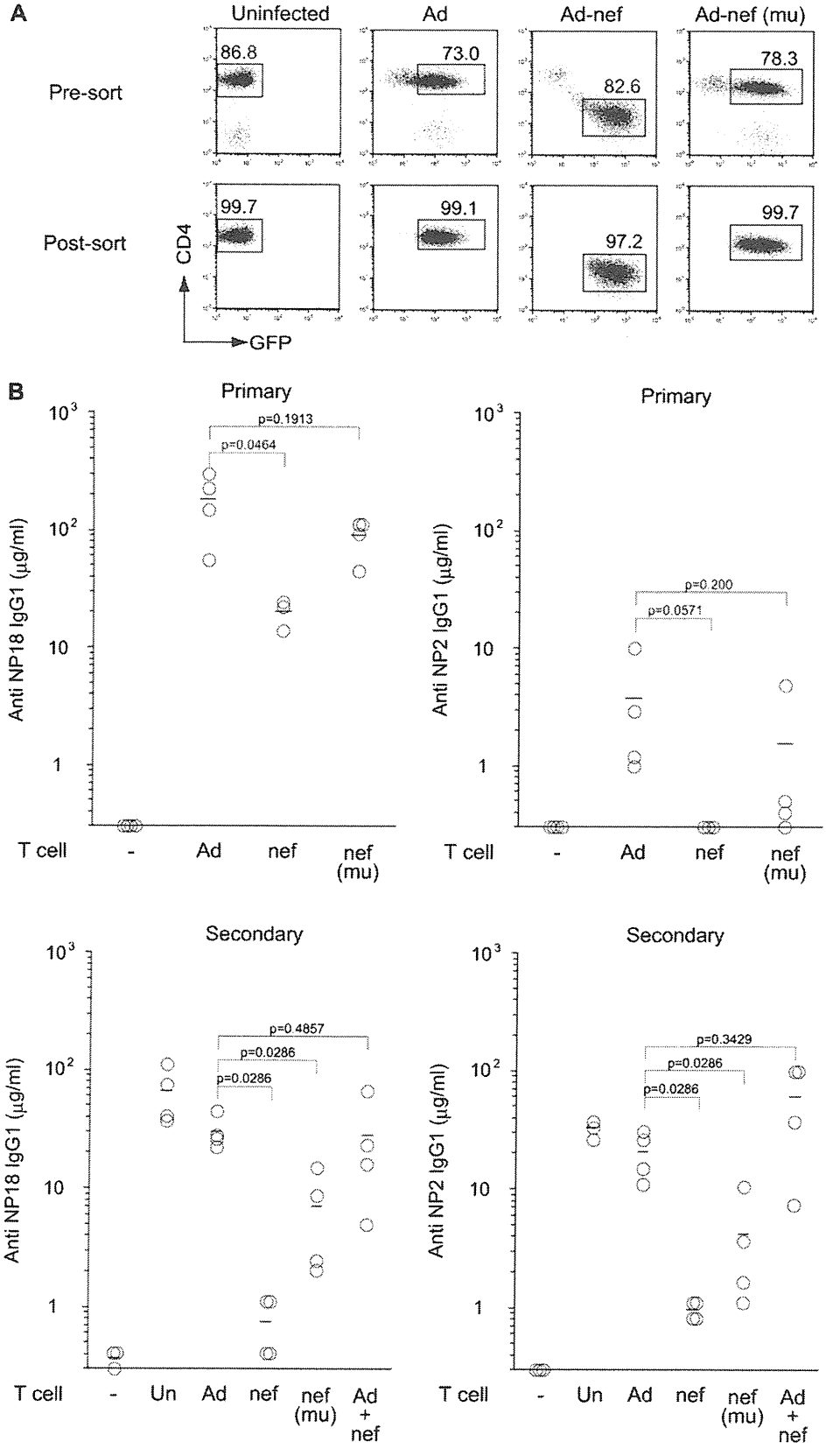

Fig. 3. Nef affects primary and memory B-cell response. (A) OVA-specific/CD4$^+$ T cells were purified from dTg mice and infected with Ad-nef, Ad-nef (mu) and Ad, followed by FACS purification (Post-sort). Numbers in plots indicate percent of GFP$^-$ uninfected cells and GFP$^+$ cells before (Pre-sort) and after purification (Post-sort). (B) Purified GFP$^+$ T cells (3×10^4) were transferred into CB17-scid mice, together with

with NP-OVA in alum for the primary response or soluble NP-OVA for the secondary response (Fig. 3B).

The results show that Nef expression in T cells reduced the level of anti-NP IgG1 serum antibodies by ~10-fold (NP_{18}; $P = 0.0464$, NP_2; $P = 0.0571$) in the primary response (Fig. 3B), whereas when the T cells were infected with Ad-Nef (mu), which does not down-regulate CD4 (Fig. 3A), the response was close to the control level (NP_{18}; $P = 0.1913$, NP_2; $P = 0.200$). As shown in Fig. 3(B), the impact of Nef on the secondary response was even more dramatic; there was a 30- to 40-fold reduction in both total and high-affinity anti-NP IgG1 antibodies (NP_{18}; $P = 0.0286$, NP_2; $P = 0.0286$). Reconstitution with equal numbers of non-infected and Nef-expressing OVA-specific $CD4^+$ T cells normalized the secondary adoptive response (NP_{18}; $P = 0.4857$, NP_2; $P = 0.3429$), excluding the possibility that Nef expression was generating suppressor T cells. Expression of the Nef mutant that was unable to down-modulate CD4 also reduced the secondary response (NP_{18}; $P = 0.0286$, NP_2; $P = 0.0286$), although the magnitude of the reduction was less than that induced by expression of wild-type Nef. These results demonstrate that Nef expression in peripheral T cells markedly diminishes their helper activity for the secondary IgG1 response and that this defect was only partially associated with the Nef-induced CD4 down-modulation. By contrast, this CD4 down-regulation appeared to be even more important for the reduced primary IgG1 response. These findings underscore the differential regulation in the primary and memory B-cell response. Thus, Nef affects helper T-cell activities in the primary and secondary response through different processes with different CD4 down-modulation susceptibility.

Discussion

In the present study, we have examined the consequence of Nef expression in primary splenic T cells. In order to avoid complications arising from expression of Nef early in T-cell development, e.g. lymphopenia, we established a double transgenic mouse (dTg), which expresses human CAR adenovirus receptor and an OVA-specific T-cell receptor that recognizes the OVA peptide on APC with high affinity under MHC Class II $I\text{-}A^d$-restriction. OVA-specific/$CD4^+$ T cells were purified from the spleen of dTg mice and infected with a recombinant adenovirus vector encoding Nef and GFP, followed by purification of GFP^+ cells using flow cytometry. To promote efficient introduction of the adenovirus vector into resting T cells, they were cultured for 2 days in the presence of the vector and IL-7, which is known to be important for survival of naive and memory T-cell populations (16). Neither naive nor memory $CD4^+$ T cells proliferate in response to IL-7, but they progress into the G_{1b} stage of the cell cycle (17). Thus, the present system allowed us to study the role of Nef in resting T cells in response to antigen-specific stimulation *in vitro* and *in vivo*.

During HIV-1 infection, the virus enters resting $CD4^+$ T cells and Nef is expressed even before the virus is integrated (1). It has been previously suggested that Nef expression in resting human T cells enhances IL-2 production upon activation by TCR cross-linking (1). This led to the proposal that Nef may enhance TCR signaling pathways that could help virus replication in partially stimulated T cells. In line with this viewpoint, it has been reported that Nef in human leukemic T cell lines and $CD4^+$ T-cell lines established from PBMC enhanced TCR signaling pathways and activated IL-2 production upon stimulation with TCR/CD28 or mitogens (2–5). In addition, Nef affects activation of murine T-cell hybridomas stimulated with anti-CD3 mAb (23), suggesting that the effect of Nef is not species specific.

In striking contrast, the present study demonstrates that Nef significantly reduces OVA-specific T-cell activation *in vitro* as defined by reduced proliferation and cytokine production, including IL-2 and IFNγ, but not completely. Furthermore, we demonstrate for the first time that Nef expression in OVA-specific resting T cells in the periphery reduced their ability to help anti-NP/$IgG1^+$ primary and secondary antibody responses in adoptive hosts after immunization with NP-OVA. In addition, in agreement with a previous *in vitro* analysis (7, 15), our *in vivo* results support the notion that Nef impairs the physiological recruitment of lymphocytes from the blood into the secondary lymphoid tissues after primary immunization, which promotes efficient antigen presentation and immune responses. Thus, Nef expressed in T cells at the early cell cycle stage impairs multiple functions in their subsequent antigen-specific response *in vivo*.

Why is the Nef-associated T-cell hyperresponse previously reported not detected in the present studies? The discrepancy does not reflect the differences in pathogenesis in *Nef* alleles (24) because the previous transgenic mouse models (8–10) and the present studies used the same NL4-3 Nef for characterization of the role of Nef protein in the immune system. Furthermore, the activation phenotype of T cells *in vitro* was induced by Nef proteins, irrespective of their alleles, including NL4-3 Nef (2–5). The discrepancy could be due to the cell state in the previous studies caused by transient over-expression of the protein in either the Jurkat T-cell line or in an activated human $CD4^+$ T-cell line established from PBMC (2–7). Another possible explanation is that previously reported assays utilized different TCR stimuli; the cells were stimulated by strong TCR ligation using immobilized antibodies (2–5). Such strong TCR ligation by antibodies forms stable TCR aggregates associated with the signaling complex (25). However, TCR stimulation with APC-presented antigen peptide forms an immunological synapse (IS) at the

---

B cells (5×10^6) which were enriched from the pooled spleens of either naive or 4-hydroxyl-3-nitrophenylacetyl-conjugated chicken γ-globulin (NP-CGG)-primed mice using a MACS system, followed by challenge with 100 μg of NP-OVA in alum (primary) or 25 μg of soluble NP-OVA (secondary). Serum anti-NP antibody titers were estimated by ELISA assays at day 7 after challenge using NP_2-BSA and NP_{18}-BSA as coating antigens. The relative affinity of anti-NP antibodies was estimated by calculating the ratio of anti-NP_2/anti-NP_{18} antibody. Representative data from two independent experiments is shown. Bars represent the mean of each group.

T-cell APC interface, facilitating signaling through TCR recognizing the peptide-loaded MHC molecules (26). The formation of IS was impaired *in vitro* by HIV-1 infection in a Nef-dependent manner (27), providing an explanation for the present results that Nef lowers the cognate interaction strength between T cells and APCs in antigen-specific response, thereby denying complete progression and activation of the cell cycle.

Nef affects helper T-cell activities in the primary and secondary response through different processes with different CD4 down-modulation susceptibility. However, the underlying mechanism remains obscure. In the B-cell response, antigen-activated helper T cells form a complex with B cells by interacting with several co-stimulatory molecules as well as with the TCR and peptide-loaded MHC class II molecules on B cells. As a consequence, T cells and B cells are mutually stimulated and T cells produce cytokines promoting B-cell proliferation and differentiation into antibody-forming cell (28). Therefore, it is likely that Nef-induced repression of T-cell helper activity for an antigen-specific B-cell response may also reflect an inefficient cognate interaction between T cells and B cells in the primary and secondary response.

We observed that Nef in resting murine $CD4^+$ T cells down-regulates the expression of CD4 on the cell surface, concordant with the previous results using human and murine T-cell lines (reviewed in ref. 1). It has been previously suggested that CD4 plays an important role in the activation of T cells by increasing the avidity of TCR for the peptide/MHC class II molecule and by transducing signals through the associated tyrosine kinase p56Lck (29). CD4 down-modulation significantly affects T-cell helper activity for the primary antibody response; however, it only partially affects T-cell helper function for the secondary response. On the other hand, Nef-mediated repression of antigen-specific T-cell function for the migratory capacity in the primary immune response is not the result of CD4 down-regulation. Thus, Nef affects multiple antigen-specific T-cell activities in the primary and secondary response through different processes with different CD4 down-modulation susceptibility, probably reflecting the T-cell signature and/or B-cell signature involved in the primary or the secondary antibody response.

CD4 binds to the MHC and boosts the recognition of ligand by the TCR in early T-cell activation, afforded by the IS formation (30) and functions to deliver Lck to the T-cell APC interface (31). However, signaling and co-stimulation later result in the movement of CD4 toward the periphery of the IS (30), suggesting that once Lck has been recruited to the synapse, the function of CD4 may become dispensable, allowing CD4 to leave the synapse, compatible with the idea that initial signal strength for T-cell activation may be crucial for the primary B-cell response. Nef might affect T-cell activity to form the IS with B cells, although, it remains unknown whether primary and memory B-cell responses require the same co-receptor molecules for T-cell interaction or if they need help from the same subset of T cells. Further analysis is needed to clarify how memory and naive B-cell responses are differentially regulated.

In summary, the present results support a model in which Nef expressing HIV-1 infected $CD4^+$ T cells fail to attain multiple functions required for normal immune responses. Thus, these combined effects of Nef may not facilitate extensive HIV-1 productions by increasing the numbers of productively infected cells through T-cell activations in antigen-specific immune response.

What could be the advantages for HIV-1 to inhibit proliferation and multiple T-cell functions required for immune response? Of note, Nef does not completely abrogate T-cell activation upon stimulation, as defined by expression of activated cell surface markers and a low level of cytokine production, which may allow a replicative advantage for the virus (reviewed in ref. 32). In activated $CD4^+$ T cells, viral replication is efficient and cytopathic (reviewed in ref. 32), though rapid death of infected cells may limit the production of the virus. By lowering the T-cell activity, Nef might facilitate a lowered level of viral spread and an increased infected T-cell life span by avoiding viral cytopathic effects. These cells may decay more slowly *in vivo* relative to activated cells, leading to vital consequences for the pathogenic outcome of infection in humans.

Supplementary data

Supplementary data are available at *International Immunology* Online.

Funding

This work was supported by a grant for Research on HIV/AIDS from the Ministry of Health, Labor and Welfare, Japan and in part by RIKEN (K94-34200 to T.T.).

Acknowledgements

We thank Drs Aya Isogai for constructing the nef (mu) plasmids and Peter Burrows for critically reviewing this manuscript and Ms Yoko Nakamura and Eri Watanabe for technical assistance.

References

1 Fackler, O. T. and Baur, A. S. 2002. Live and let die: Nef functions beyond HIV replication. *Immunity* 16:493.
2 Wang, J. K., Kiyokawa, E., Verdin, E. and Trono, D. 2000. The Nef protein of HIV-1 associates with rafts and primes T cells for activation. *Proc. Natl Acad. Sci. USA* 97:394.
3 Simmons, A., Aluvihare, V. and McMichael, A. 2001. Nef triggers a transcriptional program in T cells imitating single-signal T cell activation and inducing HIV virulence mediators. *Immunity* 14:763.
4 Manninen, A. and Saksela, K. 2002. HIV-1 Nef interacts with inositol trisphosphate receptor to activate calcium signaling in T cells. *J. Exp. Med.* 195:1023.
5 Fortin, J. F., Barat, C., Beausejour, Y., Barbeau, B. and Tremblay, M. J. 2004. Hyper-responsiveness to stimulation of human immunodeficiency virus-infected $CD4^+$ T cells requires Nef and Tat virus gene products and results from higher NFAT, NF-κB, and AP-1 induction. *J. Biol. Chem.* 279:39520.
6 Campbell, D. J., Kim, C. H. and Butcher, E. C. 2003. Chemokines in the systemic organization of immunity. *Immunol. Rev.* 195:58.
7 Janardhan, A., Swigut, T., Hill, B., Myers, M. P. and Skowronski, J. 2004. HIV-1 Nef binds the DOCK2-ELMO1 complex to activate rac and inhibit lymphocyte chemotaxis. *PLoS Biol.* 2:E6.
8 Hanna, Z., Kay, D. G., Rebai, N., Guimond, A., Jothy, S. and Jolicoeur, P. 1998. Nef harbors a major determinant of pathogenicity for an AIDS-like disease induced by HIV-1 in transgenic mice. *Cell* 95:163.
9 Rahim, M. M., Chrobak, P., Hu, C., Hanna, Z. and Jolicoeur, P. 2009. Adult AIDS-like disease in a novel inducible human immunodeficiency virus type 1 Nef transgenic mouse model:

$CD4^+$ T-cell activation is Nef dependent and can occur in the absence of lymphophenia. *J. Virol.* 83:11830.
10 Koenen, P. G., Hofhuis, F. M., Oosterwegel, M. A. and Tesselaar, K. 2007. T cell activation and proliferation characteristic for HIV-Nef transgenic mice is lymphopenia induced. *J. Immunol.* 178:5762.
11 Wan, Y. Y., Leon, R. P., Marks, R. *et al.* 2000. Transgenic expression of the coxsackie/adenovirus receptor enables adenoviral-mediated gene delivery in naive T cells. *Proc. Natl Acad. Sci. USA* 97:13784.
12 Murphy, K. M., Heimberger, A. B. and Loh, D. Y. 1990. Induction by antigen of intrathymic apoptosis of $CD4^+CD8^+TCR^{lo}$ thymocytes *in vivo*. *Science* 250:1720.
13 Kanegae, Y., Makimura, M. and Saito, I. 1994. A simple and efficient method for purification of infectious recombinant adenovirus. *Jpn. J. Med. Sci. Biol.* 47:157.
14 Takahashi, Y., Ohta, H. and Takemori, T. 2001. Fas is required for clonal selection in germinal centers and the subsequent establishment of the memory B cell repertoire. *Immunity* 14:181.
15 Choe, E. Y., Schoenberger, E. S., Groopman, J. E. and Park, I. W. 2002. HIV Nef inhibits T cell migration. *J. Biol. Chem.* 277:46079.
16 Marrack, P. and Kappler, J. 2004. Control of T cell viability. *Annu. Rev. Immunol.* 22:765.
17 Dardalhon, V., Jaleco, S., Kinet, S. *et al.* 2001. IL-7 differentially regulates cell cycle progression and HIV-1-based vector infection in neonatal and adult $CD4^+$ T cells. *Proc. Natl Acad. Sci. USA* 98:9277.
18 Craig, H. M., Pandori, M. W. and Guatelli, J. C. 1998. Interaction of HIV-1 Nef with the cellular dileucine-based sorting pathway is required for CD4 down-regulation and optimal viral infectivity. *Proc. Natl Acad. Sci. USA* 95:11229.
19 Dubey, S., Khalid, M., Wesley, C., Khan, S. A., Wanchu, A. and Jameel, S. 2008. Downregulation of CCR5 on activated CD4 T cells in HIV-infected Indians. *J. Clin. Virol.* 43:25.
20 Venzke, S., Michel, N., Allespach, I., Fackler, O. T. and Keppler, O. T. 2006. Expression of Nef downregulates CXCR4, the major coreceptor of human immunodeficiency virus, from the surfaces of target cells and thereby enhances resistance to superinfection. *J. Virol.* 80:11141.
21 Zajac, A. J., Blattman, J. N., Murali-Krishna, K. *et al.* 1998. Viral immune evasion due to persistence of activated T cells without effector function. *J Exp Med.* 188:2205.
22 Zhang, J. *et al.* 2000. Regulation of fas ligand expression during activation-induced cell death in T cells by p38 mitogen-activated protein kinase and c-Jun NH2-terminal kinase. *J. Exp. Med.* 191:1017.
23 Rhee, S. S. and Marsh, J. W. 1994. HIV-1 Nef activity in murine T cells. CD4 modulation and positive enhancement. *J. Immunol.* 152:5128.
24 Priceputu, E., Hanna, Z., Hu, C. *et al.* 2007. Primary human immunodeficiency virus type 1 nef alleles show major differences in pathogenicity in transgenic mice. *J. Virol.* 81:4677.
25 Bunnell, S. C., Hong, D. I., Kardon, J. R. *et al.* 2002. T cell receptor ligation induces the formation of dynamically regulated signaling assemblies. *J. Cell Biol.* 158:1263.
26 Dustin, M. L. 2009. The cellular context of T cell signaling. *Immunity.* 30:482.
27 Thoulouze, M. I., Sol-Foulon, N., Blanchet, F., Dautry-Varsat, A., Schwartz, O. and Alcover, A. 2006. Human immunodeficiency virus type-1 infection impairs the formation of the immunological synapse. *Immunity* 24:547.
28 Pereira, J. P., Kelly, L. M. and Cyster, J. G. 2010. Finding the right niche: B-cell migration in the early phases of T-dependent antibody responses. *Int. Immunol.* 22:413.
29 Veillette, A., Bookman, M. A., Horak, E. M., Samelson, L. E. and Bolen, J. B. 1989. Signal transduction through the CD4 receptor involves the activation of the internal membrane tyrosine-protein kinase p56lck. *Nature* 338:257.
30 Krummel, M. F., Sjaastad, M. D., Wulfing, C. and Davis, M. M. 2000. Differential clustering of CD4 and CD3ζ during T cell recognition. *Science* 289:1349.
31 Holdorf, A. D., Lee, K. H., Burack, W. R., Allen, P. M. and Shaw, A. S. 2002. Regulation of Lck activity by CD4 and CD28 in the immunological synapse. *Nat. Immunol.* 3:259.
32 Stevenson, M. 2003. HIV-1 pathogenesis. *Nat. Med.* 9:853.

第 42 回日本小児感染症学会ミート・ザ・エキスパーツ

抗体検査：目的・結果・次にすることは

庵原俊昭*

要旨　抗体検査法は，測定原理から血清を 2 倍階段希釈して測定する方法と一定の濃度に希釈後測定する方法の 2 種類がある．測定方法による互換性がわかるようにWHO は抗体価を国際単位で表示することを勧めている．抗体検査は感染症の診断と各感染症の免疫保有状態の検索に用いられる．各感染症とも抗体陽性抗体価，発症予防抗体価，感染予防抗体価は異なっており，発症予防抗体価でワクチン予防可能疾患の感染対策が行われている．

はじめに

抗体検査は，感染症の診断，各感染症に対する免疫保有状態の検索，ワクチン開発における有効性の指標に広く用いられている．抗体検査を有効に利用するためには，抗体測定方法の原理を理解し，結果を感染症の病態に応じて弾力的に解釈することが大切である．抗体検査の原理および抗体検査の臨床応用について解説する．

Ⅰ．抗体測定方法の原理と測定方法（表 1）

血清抗体測定方法は測定原理から大きく分けて 2 種類に分類される[1]．一つは「階段血清希釈法」と呼ぶ方法で，血清を 2 倍階段希釈後，ウイルスまたは抗原を添加し，所定の時間反応させたあと，反応結果である抗原抗体複合体を粒子の凝集〔粒子凝集法（PA）：ウイルス抗原を付着させたラテックス粒子などを使用〕，反応後添加した蛍光標識させた二次抗体の蛍光〔間接蛍光抗体法（IFA）〕，反応後添加したヒト O 型赤血球の凝集〔免疫付着赤血球凝集法（IAHA）〕などで判定する方法と，反応後に残ったウイルスの増殖〔中和法（NT）〕，残った赤血球凝集抗原を反応後添加した動物赤血球の凝集〔赤血球凝集抑制法（HI）〕，抗原抗体反応時に添加した補体の残存による反応後

表 1　抗体測定の原理と測定方法

1．階段血清希釈法
　1）抗原抗体反応を検出
　　・粒子凝集法（PA）
　　・間接蛍光抗体法（IFA）
　　・免疫付着赤血球凝集法（IAHA）
　2）反応せずに残ったウイルス，抗原または補体を検出
　　・中和法（NT）
　　・赤血球凝集抑制法（HI）
　　・補体結合法（CF）
2．一定濃度血清希釈法
　検量線から算出
　　・酵素免疫法（EIA）
　　・放射性免疫測定法（RIA）
　　・化学発光免疫測定法（CLIA）
　　・ラテックス凝集比濁法（LA）

階段血清希釈法は手作業で行われ，目視で判定される．抗体価は「倍」で表示される．一方，一定濃度血清希釈法は機械化が可能であり，多数の検体を測定するのに適している．抗体価は「単位」で表示される．

Key words：抗体，IgM 抗体，抗体有意上昇，抗体陽性，発症予防抗体価
* 国立病院機構三重病院小児科
〔〒 514-0125 津市大里窪田町 375〕

添加した赤血球の溶血〔補体結合法（CF）：溶血素で標識したヒツジ赤血球（感作赤血球）を添加〕で判定する方法などがある．HI 抗体測定に用いる動物赤血球は，測定するウイルスによって異なっている．判定基準も，反応を 50%認めた血清希釈倍数で表示する場合と，100%認めた血清希釈倍数で表示する場合とがあり，判定基準により抗体価は異なってくる．測定の多くの部分は手作業で行われ，判定も目視で行われる．抗体価は通常「倍」で表示される．

もう一つの血清抗体測定方法は「一定濃度血清希釈法」と呼ぶ方法で，複数の標準血清の測定値から検量線を作製し，測定値から抗体価を求める方法である．血液生化学検査やホルモン検査などと同じ測定原理である．一定濃度に希釈した血清を一定濃度のウイルス抗原と反応させたあと，抗原に付着した抗体量を，発色色素で標識された二次抗体で測定する方法〔酵素免疫法（EIA)〕，ラジオアイソトープで標識された二次抗体で測定する方法〔放射性免疫測定法（RIA)〕，化学発光物質で標識された二次抗体で測定する方法〔化学発光免疫測定法（CALI)〕，ラテックス粒子の濁度でみる方法〔ラテックス凝集比濁法（LA)〕などがある．手作業で行われるときもあるが測定の機械化が可能であり，多数の検体を測定するのに適している．測定結果は連続した数字で表示される．なお，反応する抗原量が限定されているため，高い抗体価は低く表示される傾向があり，ときに測定限度以上になることがある．測定限度以上になった検体は，測定値が検量線にフィットするようさらに希釈して測定する．測定された抗体価は，通常「単位」で表示される．

II．抗体価の分布と標準化

「倍」で表示された抗体価も「単位」で表示された抗体価も，集団における陽性抗体価は対数変換すると正規分布する．抗体価を群間比較するとき，「倍」で表示される抗体価は，習慣上 2 を底とする対数に変換後検定を行い，「単位」で表示される抗体価は，10 を底とする対数または 2 を底とする対数に変換後検定を行っている．

いろいろな測定方法で測定された抗体価を比較するためには，統一された抗体価表示が必要である．世界保健機関（WHO）は血清抗体価表示の統一化を目指し，値付けした標準血清を作製し，B 型肝炎ウイルス（HBV)，麻疹，風疹などの抗体価を国際単位（IU）で表示することを求めている．わが国では HBs 抗体は国際単位が使用されており，風疹抗体価および麻疹抗体価も国際単位表示への動きがある．ちなみに，風疹 HI 抗体価 8 倍は 8 IU に，風疹 EIA 抗体価 4.0 EIA 価は 8 IU に相当し，麻疹 NT 抗体 256 倍は 10,000 mIU に相当する[2]．

III．抗体の有意上昇

抗体価の有意上昇とは，測定誤差以上の抗体価の上昇である．2 倍階段血清希釈法で測定された抗体価では，測定抗体価の上下 2 倍（1 管の差）までの抗体価は誤差範囲であり，4 倍（2 管）以上の上昇が測定誤差以上の上昇である．一方，一定濃度血清希釈法で測定される抗体価では誤差範囲の幅は狭く，2 倍以上の抗体上昇は誤差範囲以上の上昇である．

IV．抗体による感染症の診断

感染症診断の基本は，病巣からの病原体検出（ウイルス分離，細菌培養同定）である[3]．その他の診断方法として，ウイルス蛋白またはウイルス遺伝子の検出，血清 IgM 抗体の検出，血清抗体（IgG 分画）の有意上昇がある．ウイルス血清抗体価は，体内で増殖したウイルス量に応じた免疫反応であり，時間の経過とともに IgG 抗体も IgM 抗体も上昇する．1 回の血清 IgM 抗体検査で感染症を診断するためには，発症 48 時間以降に測定することが望ましい．なお，血清 IgM 抗体はときに非特異陽性を示すことがあり，臨床経過からその感染症が否定的な場合は再検すべきである．非特異陽性の場合は，時間の経過による IgM 抗体の上昇が認められない．伝染性紅斑，突発性発疹，デング熱では麻疹 IgM 抗体が非特異陽性になる[4,5]．

ウイルス抗体は，ワクチン後の感染および再感染の診断にも用いられる．このような症例では，症状出現時にすでに二次免疫応答が始まっており，IgG 抗体は上昇し，ウイルス増殖の程度に応

表 2　ムンプスウイルス分離陽性例の急性期 IgM・IgG 抗体価

症例	年齢	V 歴	病日	V 分離	IgM 抗体	IgG 抗体
1	9	−	1	+	3.71	2.1
2	6	−	1	+	11.99	14.0
3	9	−	1	+	0.16	2.5
4	5	−	1	+	3.26	7.5
5	9	−	3	+	12.55	8.5
6	4	−	3	+	14.82	16.3
7	4	−	3	+	13.05	12.1
8	5	+	1	+	0.55	26.6
9	6	+	1	+	1.26	41.9
10	5	+	1	+	0.74	29.1
11	6	+	2	+	0.44	35.7
12	6	+	2	+	0.40	2.2
13	5	+	3	+	1.43	31.4

じて IgM 抗体が検出される．**表 2** にムンプス初感染時およびワクチン後のムンプス発症時の抗体パターンを示した．表 2 の症例 12 は一次性ワクチン不全（primary vaccine failure：PVF）であり，他の 5 例は二次性ワクチン不全（secondary vaccine failure：SVF）である．なお，デンカ生研のムンプス IgM 抗体検査試薬や麻疹 IgM 抗体検査試薬で測定された IgM 抗体価は定量性があり，14.0〜15.0 抗体指数が測定限界である．この抗体価以上の抗体価を測定するときは，血清を希釈して再測定する必要がある．

ウイルス抗体の検査結果を読むときは，時間的経過を考慮することが大切である．また，地域での流行や園や学校での流行を確認することも大切である．

V．抗体陽性抗体価，発症予防抗体価，感染予防抗体価

抗体測定方法により陽性閾値は異なっており，測定感度が高い抗体測定方法を用いると，少ない抗体量まで検出できる．同じ感度の測定方法を用いても抗体量が少ないと，ときに測定方法により結果が異なることがある．このような場合は陰性として対応する．抗体判定基準に（±：同等，判定保留）を含む測定方法では，（±）と判定された場合も多くは陰性として対応する．

表 3　抗体測定方法による麻疹・風疹各種抗体価

	単位	陽性レベル	発症予防レベル	感染予防レベル
麻疹				
国際単位	mIU/ml		120〜200	500〜1,000
NT	倍	2	4	32
PA	倍	16	64	256
EIA	EIA 価	2.0	4.0	16.0
HI	倍	8	8	16
風疹				
国際単位	IU/ml	4	10	15〜25
LA	IU/ml	4	10	15〜25
HI	倍	8	16	32
EIA	EIA 価	2.0	5.0	12.5

NT：中和法，PA：粒子凝集法，EIA：酵素免疫法
HI：赤血球凝集抑制法，LA：ラテックス凝集法

ウイルスの 50%細胞変性効果（cytopathic effect：CPE）抑制を判定基準として判定された NT 抗体 2 倍は抗体陽性であるが，発症予防抗体価ではない．また，ウイルス感染症には全身性ウイルス感染症と局所性ウイルス感染症がある．全身性ウイルス感染症では，ウイルスが感染後増殖して症状が出現するまでの間に，免疫記憶細胞が誘導されていると早期に二次免疫応答が始まり，結果として発症が予防される．感染予防抗体価とは，感染したウイルスの増殖を抑制する抗体価であり，病態的には二次免疫応答を誘導させない抗体価である．全身性ウイルス感染症では，感染予防抗体価のほうが発症予防抗体価よりも高値である．

一方，局所性ウイルス感染症では，ウイルス感染後二次免疫応答による症状発現抑制よりも先に症状が出現するため，発症予防には高い抗体価が必要である．発症予防抗体価と感染予防抗体価は一致する．

感染症ごとに，また抗体測定方法ごとに抗体陽性抗体価，発症予防抗体価，感染予防抗体価は異なっている．**表 3** に麻疹および風疹の抗体測定方法による抗体陽性レベル，発症予防レベル，感染予防レベルの抗体価を示し[2,6]，**表 4** に代表的なワクチン予防可能疾患の発症予防抗体価を示した[7,8]．なお，この抗体価は多くの人の発症を予防する抗体価であり，曝露されたウイルス量が多い

表 4 代表的なワクチン予防可能疾患の発症予防レベル

ワクチン	抗体測定方法	必要な抗体価
ジフテリア	中和	0.01～0.1 IU/ml
A 型肝炎	EIA	10 mIU/ml
B 型肝炎	EIA	10 mIU/ml
Hib 結合型	EIA	0.15 μg/ml
インフルエンザ	HI	40 倍
日本脳炎	中和	10 倍
麻疹	マイクロ中和	120 mIU/ml
ムンプス		not defined
百日咳	EIA（PT）	5 単位
肺炎球菌	EIA	0.20～0.35 μg/ml (小児)
	opsonophagocytosis	8 倍
ポリオ	中和	4～8 倍
狂犬病	中和	0.5 IU/ml
ロタウイルス		not defined
風疹	免疫沈降	10～15 IU/ml
破傷風	中和	0.1 IU/ml
水痘	FAMA	64 倍
	gp ELISA	5 IU/ml
黄熱	中和	5 倍

（文献 6）より引用，一部改変）

と，発症予防のためにはこの抗体価よりも高い抗体価が必要である．また，抗体価が高いほど発症予防効果は優れている．

感染予防には液性免疫だけではなく，粘膜免疫や細胞性免疫も働くため，発症予防抗体価よりも低い抗体価でも発症しないことがあり，抗体価だけで発症予防を判断することは困難である．しかし，抗体価は容易に測定できるため，一般には抗体測定により免疫保有状態の検索が行われている．なお，免疫保有状態の検索には，感受性の高い抗体測定方法を用いるべきである．費用を考えなければ EIA 法が優れており，CF 法は用いるべきではない．発症予防レベル以下の人にはワクチン接種が勧められる．

VI. Low responder（低抗体反応者）

発症予防レベル以下の抗体価の人にワクチンを接種しても，発症予防レベル以上の抗体価上昇が認められないときがある．生ワクチンの場合は，細胞性免疫などでウイルス増殖が抑制される人であり，発症が予防される人である．また B 型肝炎ワクチンなどの不活化ワクチンでも，規定通りのプライミングとブースティングを行っても抗体価が上昇しない人がいる．このような人は low responder と呼ばれ，遺伝的因子が関与している．Low responder は感染しても発症しない人と考えられている．

2 回麻疹ウイルスを含むワクチンを接種しても，発症予防レベル以上の抗体価に達しない人がいる．このような人への追加接種は理論上不要であるが，社会的要因によって 3 回目の接種が勧められる場合がある．

VII. 感染後・ワクチン後の免疫持続

1781 年に麻疹が流行し，その後麻疹流行がなかった Faroe 島で 1846 年に麻疹が流行したとき，1781 年の流行を経験した 65 歳以上の人たちは麻疹を発症しなかったが，64 歳以下の人たちは麻疹を発症した．この報告から麻疹は一度かかると生涯免疫が持続すると考えられていた．

一方，麻疹ワクチンを受けた一部の人たちが麻疹流行時に軽症の麻疹（修飾麻疹）を発症することがあり，すべの人が生涯発症予防レベル以上の抗体価を持続することがないことが示され，先進国では SVF 予防に麻疹ウイルスを含むワクチンの 2 回接種を行っている．しかし，2 回接種を受けた人でもまれに麻疹発症が認められている[9]．なお，麻疹だけではなく水痘やムンプスにおいても，ワクチン後の自然罹患例の臨床経過は，ワクチンを受けていない自然罹患例と比べ軽症である．

SVF の病態を図に示した．感染予防レベルの抗体価がないためウイルスは感染し，感染したウイルス増殖と同時に初回ワクチン接種により誘導された免疫細胞が刺激され二次免疫応答は開始するが，二次免疫応答により産生される抗体などの特異免疫が不十分なため，発症抑制が間に合わず症状は出現する．しかし，症状出現後も免疫応答は働き，結果として症状が早期に消失し軽症化する．

全身性ウイルス感染症に対する生ワクチン接種後の抗体価の半減期は 3～4 年である．ワクチン後の抗体の持続は自然感染による免疫賦活（自然

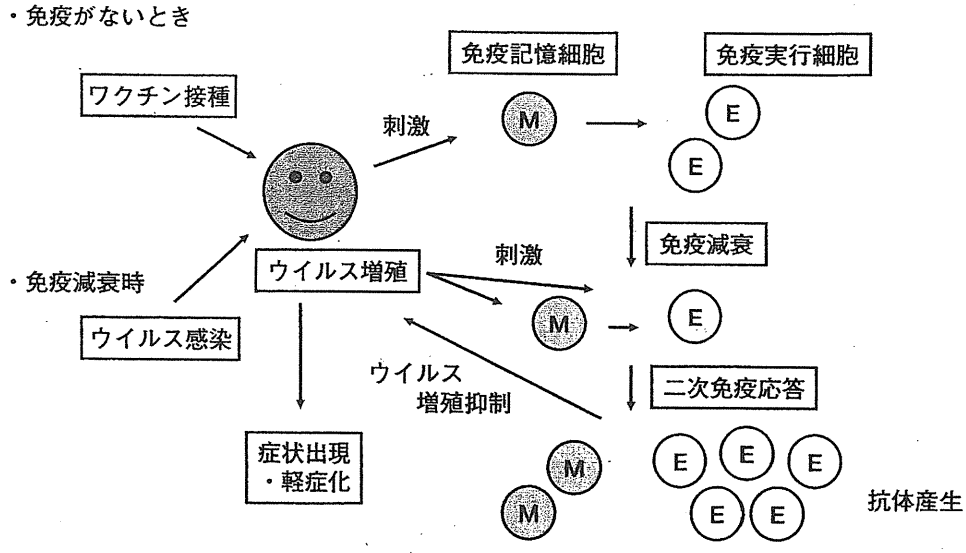

図　二次性ワクチン不全（SVF）の病態

高い抗体価だと感染が予防され，中等度の抗体価だと二次免疫応答により発症が予防され，低い抗体価だと発症するが軽症化する（修飾感染）．

ブースター）であり，自然ブースターがなければ抗体価はそのまま減衰を続け，多くの人は流行時に再感染すると考えられていた．一方，長期間の経年的な抗体価測定結果から，流行がなくても抗体価は長期に維持されるという報告もある[10,11]．Amanna らが示す抗体価の半減期を**表 5** に示した[10]．

これらの結果から，B 細胞には memory B cells dependent short-lived plasma cells（記憶 B 細胞依存性短命プラズマ細胞）と memory B cells dependent long-lived plasma cells（記憶 B 細胞依存性長命プラズマ細胞）の 2 種類があると考えられている．ワクチン後の抗体価の半減期や Faroe 島の経験から，自然感染後やワクチン後早期の抗体産生細胞は主として前者が，自然感染後やワクチン後数年以降の抗体産生細胞は主として後者が関係していると考えると理解しやすい．また，自然感染後やワクチン後の抗体価が高いほど長期に維持される抗体価も高く，自然ブースターがなくても高い抗体価が維持されると考えると，Faroe 島の経験が説明できる．実際，麻疹流行規模が小さくなった近年でも，麻疹ワクチン世代と比べ麻疹自然感染世代の麻疹抗体価は高値である[12]．

表 5　抗体の半減期

抗原	予防レベル (IU/ml)	抗体半減期（年） 男性	女性
破傷風	0.01	12（10～16）	10（8～14）
ジフテリア	0.01	26（17～51）	14（8～42）
麻疹	0.2	369（67～∞）	減衰なし（74～∞）
風疹	10	85（43～∞）	190（35～∞）
水痘	NA	63（28～∞）	41（23～212）
ムンプス	NA	124（53～∞）	減衰なし（89～∞）
天然痘	3.8	99（48～∞）	85（31～∞）

NA：not applicable
Memory B cells dependent short-lived plasma cells による抗体産生と Memory B cells dependent long-lived plasma cells による抗体産生
（文献 10）より引用，一部改変）

Ⅷ．インフルエンザ抗体価

ウイルス抗体価測定の基本は NT 法である．しかし，インフルエンザウイルスが分離された当時，効率的に増殖できる培養細胞がなく，インフルエンザウイルス増殖には発育鶏卵が用いられていた．このため NT 抗体の測定が困難であり，HI 法が広く用いられた．インフルエンザウイルスが効率的に増殖する MDCK 細胞がみつかった現在でも，手間と時間がかかる NT 法よりも HI 法がイ

表 6 インフルエンザ HI 抗体測定結果の解釈

1）インフルエンザワクチン株

シーズン	A/H1N1	A/H3N2	B
2008/09	ブリスベン/59/2007	ウルグアイ/716/2007	フロリダ/4/2006
2009/10	ブリスベン/59/2007	ウルグアイ/716/2007	ブリスベン/60/2008
2010/11	カリフォルニア/7/2009	ビクトリア/210/2009	ブリスベン/60/2008

2）同じ血清の各シーズンの HI 抗体価

HI 抗体価	A/H1N1	A/H3N2	B
2009/10 シーズン	80	160	40
2010/11 シーズン	<10	10	40

3）説明：インフルエンザ HI 抗体価は，そのシーズンに用いられるワクチン株から作製した HA 抗原を用いて測定される．2009/10 シーズンと 2010/11 シーズンを比較すると，A/H1N1 は抗原性が大きく異なる株が，A/H3N2 も抗原性が 16 倍異なる株が用いられている．このため，同じ血清でも抗原性が変異すると，抗体価も相対的に低下する．

ンフルエンザ抗体測定に広く用いられている．HI 法 10 倍は測定原理上抗体陽性であるが発症予防効果がないため，50％の発症予防効果がある HI 抗体 40 倍を，インフルエンザでは抗体陽性閾値と定義している．インフルエンザ抗体陽性率は HI 抗体 40 倍以上の人の割合である．

インフルエンザウイルスは変異が早いウイルスであり，世界各地から分離されたインフルエンザウイルスの抗原性の検討から毎年の南半球および北半球のインフルエンザワクチン株が決定されている．毎年のインフルエンザワクチンには A/H1N1，A/H3N2，B 型の 3 種類のウイルスが含まれており，インフルエンザ抗体価はその年のワクチン株由来のヘマアグルチニン（HA）抗原を用いて測定されている．この結果，抗原性が異なるワクチン株が導入されると，同じ血清でも前年測定した抗体価と当年測定した抗体価が異なる結果となる（**表 6**）．ちなみに 2010/11 シーズンの A/H1N1 と A/H3N2 のワクチン株の抗原性は，2009/10 シーズンと大きく異なっている．なお，ワクチン後のインフルエンザ抗体価は，ウイルスの抗原性がかわらなければ半年～1 年後に約 1/2 に低下する．また，B 型の HA 抗原性は低いため，HI 抗体価は低めに表示される．

ま と め

ウイルス抗体を中心に抗体検査の原理，抗体検査結果の解釈，および抗体の持続について解説した．抗体価は免疫反応や免疫力の一つの指標であり，単に抗体価の数字にとらわれることなく臨床経過全体で判断することが大切である．

文 献

1）庵原俊昭：ウイルス感染症の診断．小児科診療 68：1992-1999，2005
2）庵原俊昭，他：風疹・麻疹抗体測定法の標準化に関する研究：抗体測定方法の互換性と感染予防レベルの検討．ウイルス感染症の体外診断薬の再評価に関する基盤整備に関する研究（研究代表者：小林和夫）平成 21 年度総括・分担研究報告書，2010，19-25
3）庵原俊昭：小児感染症の診断．小児感染症学（岡部信彦編）．診断と治療社，東京，2007，21-27
4）田中敏博，他：伝染性紅斑の成人患者における血清中の麻疹ウイルス IgM 抗体価の変動．病原微生物検出情報 31：268-269，2010
5）佐藤 弘，他：デング熱および突発性発疹と考えられる症例における麻疹 IgM 抗体陽性例．病原微生物検出情報 31：269-271，2010
6）庵原俊昭：ワクチンと免疫．小児保健研究 69：830-832，2010

7) Plotkin SA : Correlates of protection induced by vaccination. Clinical and Vaccine Immunology 17 : 1055-1065, 2010
8) Plotkin SA : Correlates of vaccine-induced immunity. Clin Infect Dis 47 : 401-409, 2008
9) Chen T, et al : Measles outbreak associated with an international youth sporting event in the United States, 2007. Pediatr Infect Dis J 29 : 794-800, 2010
10) Amanna IJ, et al : Duration of humoral immunity to common viral and vaccine antigens. N Engl J Med 357 : 1903-1915, 2007
11) Slifka MK, et al : Long-term humoral immunity against viruses : revisiting the issue of plasma cell longevity. Trends Microbiol 4 : 394-400, 1996
12) Ihara T : The strategy for prevention of measles and rubella prevalence with measles-rubella (MR) vaccine in Japan. Vaccine 27 : 3234-3236, 2009

Antibody examination : the purpose, the results, and the next steps

Toshiaki IHARA

Department of Pediatrics, National Hospital Organization, Mie National Hospital

According to the manners of serum dilution, two methods of detecting the antibodies are present. One is that the serially two-fold diluted sera are used, and the other is that sera are used after settled dilution. World Health Organization recommends antibody titers are represented by international units (IU) for comparison with the titers detected by different methods. Antibody examination is widely used to make diagnosis of infectious diseases and to detect seropositivity. The positive titers, symptom preventable titers, and infection preventable titers are different in each infectious disease. The symptom preventable titers are indicated to prevent the diseases by vaccination.

* * *

麻疹，風疹，水痘，ムンプスの患者に接触したときの感染予防措置はどうすればよいですか

庵原俊昭*

Ⅰ．麻疹，風疹，水痘，ムンプスの感染力

- ヒトからヒトに感染する感染症（ヒトヒト感染症）の感染力は，一人の感染した人が免疫のない周囲の人何人に感染させるかの数（基本再生産数：R_0）で表される。
- 感染症の流行を止める集団免疫率は $(1-1/R_0)\times 100$ で求められ，基本再生産数が高い感染症ほど感染力が強く，高い集団免疫率が必要である（表1）。
- また，ヒトヒト感染症では曝露された病原体の量と曝露時間が，周囲の人への感染に関係している。曝露量が多い顔と顔が向き合った状態では短時間で感染し，同じ室内にいる場合は曝露量が少ないため長時間の曝露時間が必要である。
- 水痘の場合，顔と顔が向かい合った場合は5分間，同じ部屋にいた場合は60分間で他人に感染させる[1]。基本再生産数が高い感染症ほど短時間の曝露で周囲に感染する。
- 麻疹，風疹，水痘，ムンプスにおいて感染を受けても発症しないと予測される状態は，流行時の明らかな既往，文書で証明された2回のワクチン歴，発症予防以上の抗体価の保有，である。
- 既往歴が不明の場合は免疫がないとして対応し，1回しかワクチンを受けていない人で確実な発症予防を希望する場合は，2回目のワクチン接種が勧められる。
- 麻疹，水痘などの患者との接触時の感染予防措置の原則は，生ワクチン接種可能者にはできるだけ早期にワクチンを接種し，生ワクチンが接種できない者にはγグロブリンの投与が，とくに免疫不全者では倍量の投与が考慮される。

Ⅱ．麻疹の感染予防措置

- 麻疹患者と接触後72時間以内に麻疹ワクチンを接種すれば発症予防効果が，120時間以内な

表1　麻疹・風疹・水痘・ムンプス・インフルエンザの基本再生産数・集団免疫率と感染に要する接触時間

感染症	基本再生産数	集団免疫率（%）	接触時間*	
			向かい合わせ	同室内
麻疹	16～21	90～95	NA	≧20分間
風疹	7～9	80～85	NA	NA
水痘	10	90	≧5分間	≧60分間
ムンプス	11～14	85～90	NA	NA
インフルエンザ	1.4～2.4	50	≧15分間	≧24時間†

NA：not applicable
集団免疫率≒(1－1/基本再生産数)×100
*各病原体がヒトヒト感染するために要する時間
†感染者が動いた場合。おとなしくしていると，もっと長い時間が必要

*Ihara Toshiaki
国立病院機構三重病院小児科〔〒514-0125　津市大里窪田町357〕
TEL 059-232-2531　FAX 059-232-5994　E-mail：ihara@mie-m.hosp.go.jp