in serum samples from HIV-infected adults. We report here a decrease in serum opsonic activity against *S. pneumoniae*, despite increased levels of type-specific IgG, in HIV-infected Ugandan adults.

METHODS

Subjects and serum samples. From November 1998 through March 2001, 36 patients with CAP were enrolled in our study on the day of admission to Mulago Hospital, Makerere University (Kampala, Uganda), after written informed consent had been obtained [6]. The diagnostic criteria for CAP have been described elsewhere [6, 17, 18]. Of these patients, 29 (13 men and 16 women) were infected with HIV-1, and 7 (6 men and 1 woman) were not HIV infected. From July through October 1998, 35 asymptomatic HIV-1–infected Ugandan adults (10 men and 25 women) and 23 asymptomatic HIV-uninfected Ugandan adults (17 men and 6 women) were randomly enrolled at the Joint Clinical Research Centre (Kampala) after providing informed consent [19]. Serum samples were obtained from these subjects at the time of enrollment and stored at −80°C. Neither the HIV-1–infected patients with CAP nor the asymptomatic HIV-infected subjects had received any antiretroviral therapy. HIV-1 serostatus and $CD4^+$ lymphocyte counts in peripheral blood were determined at the time of enrollment [6, 19]. For the 35 asymptomatic HIV-1–infected subjects, plasma HIV RNA loads were quantified as described elsewhere [19]. The mean $\log_{10}$ plasma HIV RNA load (±SD) of these subjects was 4.4 ± 1.0 copies/mL. The protocols of these 2 studies were reviewed and approved by the Ugandan AIDS Research Committee and the National Council for Science and Technology of Uganda. Frozen serum samples were shipped to Japan in temperature-controlled (liquid nitrogen) thermal containers. Control immune serum was obtained from healthy volunteers who had been immunized with the 23-valent pneumococcal polysaccharide vaccine.

Measurement of total IgG and serotype-specific IgG. Total IgG levels in serum were measured by laser nephelometry using purified human IgG and goat antibody to human IgG (Cappel). During the period 1998–2002, we determined the serotype of pneumococcal strains isolated from patients with CAP in Uganda. Among 29 strains, 3 strains of type 3 and 5 strains of type 9 were identified (authors' unpublished data). We therefore decided to determine the levels of type-specific IgG to type 3 and type 9 CPS (because these are the major serotypes) by ELISA, according to a method reported elsewhere [20, 21]. Ninety-six–well flat-bottom microtiter plates were coated with bicarbonate buffer containing type 3 CPS (50 μg/mL) or type 9 CPS (5 μg/mL) and incubated overnight at 4°C. Twofold serially diluted preabsorbed serum with cell-wall polysaccharide (CWPS; Statens Serum Institute, Copenhagen, Denmark) was added to the antigen-coated plates, and the plates were incubated for 30 min at room temperature [20]. After the plates were washed, alkaline phosphatase–conjugated anti–human IgG (Biosource), diluted 1:2000, was added to each well, and the plates were incubated for 30 min at room temperature. The reaction was developed by *p*-nitrophenyl phosphate (Sigma Chemicals), and the optical density at 405 nm was read. The end-point titers were expressed as the reciprocal $\log_2$ of the final dilution, giving an optical density at 405 nm of >0.016 for type 3 CPS and >0.130 for type 9 CPS.

Chemiluminescence (CL) assay and opsonophagocytic killing assay. Luminol-enhanced CL was measured as described elsewhere [22]. *S. pneumoniae* serotypes 3 (strain P97-182) and 9 (strain P20-049), both isolated from Ugandan patients with CAP, were used for this assay. Human polymorphonuclear leukocytes (PMNLs) were isolated from the peripheral blood of a healthy, HIV-uninfected volunteer and suspended in Hanks' balanced salt solution buffer with Ca^{2+} and Mg^{2+} ($HBSS^{++}$). Baby rabbit complement (Cedarlane Laboratories) was used as a complement source. The reaction mixtures contained human PMNLs (5×10^5 cells), bacterial suspension (5×10^6 cfu), 0.1 mg of luminol (5-amino-2,3-dihydro-1,4-phthallazinedione; Sigma), 25 μL of complement, and 25 μL of heat-inactivated test serum or heat-inactivated control immune serum (CS) in a total volume of 500 μL of $HBSS^{++}$ containing 1% gelatin. After the reaction mixtures had been allowed to equilibrate at 37°C for 10 min, the bacterial suspension, complement, and heat-inactivated test serum or the CS were added to activate the system. The light emission was recorded continuously for 90 min with a 6-channel Biolumat LB9505 luminometer (Berthold).

The phagocytic killing assay for the killing of type 9 *S. pneumoniae* by human PMNLs was simultaneously performed in the same manner as the CL assay, using 7% rabbit blood agar, as described elsewhere [23]. The percentage of survival (mean ± SD of 4 tests) was calculated as follows: (bacterial load after incubation/bacterial load before incubation) × 100. No significant bacterial killing was found in samples containing PMNLs alone (94.8% ± 7.4%), PMNLs and CS (81.8% ± 15.9%), or PMNLs and complement (90.5% ± 13.7%). In contrast, a significant level of bacterial killing was found in samples containing PMNLs, CS, and complement (13.8% ± 2.9%). The CL response peaked and the light emission reached 8.21×10^7 cpm in samples containing PMNLs, CS, and complement at 40 min after incubation. However, a minimal increase in light emission was found in samples containing PMNLs and CS (1.96×10^6 cpm), PMNLs and complement (1.76×10^7 cpm), and PMNLs alone (7.49×10^6 cpm) at 40 min after incubation. These results indicate that a correlation exists between opsonophagocytic killing and the luminol-enhanced CL response. We, therefore, used the CL ratio (the ratio

Table 1. Clinical characteristics and laboratory data for 64 subjects with community-acquired pneumonia (CAP) and 30 asymptomatic (AS) subjects in Uganda.

Characteristic	HIV-1–infected subjects		HIV-uninfected subjects	
	CAP (n = 29)	AS (n = 35)	CAP (n = 7)	AS (n = 23)
Age, mean years ± SD	38.0 ± 8.3	34.2 ± 6.7	39.7 ± 15.3	31.6 ± 8.9
Peripheral blood CD4⁺ cell count, mean cells/μL ± SD	151 ± 138[a]	471 ± 295	495 ± 214[b]	909 ± 250
Serum IgG level, mean mg/mL ± SD	38.0 ± 8.4[c]	38.0 ± 9.1[c]	19.9 ± 3.8	20.0 ± 4.3

[a] $P < .0001$, vs. HIV-infected or HIV-uninfected AS subjects; $P = .012$, vs. HIV-uninfected patients with CAP.
[b] $P = .002$, vs. HIV-uninfected AS subjects.
[c] $P < .001$, vs. HIV-uninfected patients with CAP or HIV-uninfected AS subjects.

of the peak CL value in the presence of test serum to the peak CL value in the presence of the CS) to evaluate serum opsonic activity. Because of the limited volume of stored serum, 2 samples from HIV-1–infected asymptomatic subjects and 15 samples from HIV-uninfected asymptomatic subjects were not available for testing of opsonic activity against type 3 strains.

Statistical analysis. The StatView statistical package (version 5.0) and SPSS software (version 10) were used for data analysis. All data were expressed as mean ± SD. Levels of serum IgG, levels of serum IgG to CPS, and serum opsonic activity were compared between the 2 groups using the unpaired Student's t test or the Mann-Whitney U test. The subject's age and peripheral blood CD4⁺ lymphocyte count, levels of serum IgG, and bacterial killing in the opsonophagocytic assay were analyzed by 1-way analysis of variance and by multiple comparison methods, including the Bonferroni-Dunn and Scheffé tests. Levels of serum IgG to CPS and serum opsonic activity among patients with CAP and asymptomatic subjects with or without HIV infection were analyzed by the Kruskal-Wallis test and Turkey's multiple comparison. The significance of the correlations was estimated using Spearman's rank correlation. Data were considered to be statistically significant when P was <.05.

RESULTS

Total IgG and type-specific IgG. The clinical and laboratory characteristics of 64 HIV-1–infected and 30 HIV-uninfected subjects are shown in table 1. Among 29 HIV-1–infected patients with CAP, 7 patients were found to be infected with *S. pneumoniae*, whereas no pneumococcal pneumonia was found among 7 HIV-uninfected patients with CAP. The peripheral blood CD4⁺ lymphocyte count of patients with CAP was significantly lower than that of asymptomatic subjects in the HIV-uninfected group ($P = .002$) and in the HIV-1–infected group ($P < .001$). Decreased CD4⁺ lymphocyte counts, however, have been found in patients with CAP during the acute phase, regardless of HIV status [24]. The total serum IgG level among HIV-1–infected subjects was higher than that among HIV-uninfected subjects (38.20 ± 8.74 vs. 19.95 ± 4.14 mg/mL; $P < .001$), which confirms previous reports of B cell activation in such individuals [8–11]. No significant difference, however, was found in total levels of serum IgG between patients with CAP and asymptomatic subjects, regardless of HIV serostatus (table 1). The titers of IgG to type 3 and type 9 strains in serum were significantly higher among the 64 HIV-1–infected subjects than among the 30 HIV-uninfected subjects (8.34 ± 0.93 vs. 7.07 ± 1.02 for type 3 and 10.13 ± 1.32 vs. 7.50 ± 1.01 for type 9; $P < .001$, for either type); total levels of serum IgG were significantly correlated with levels of serum IgG to type 3 CPS ($r = 0.684$; $P < .0001$) and type 9 CPS ($r = 0.716$; $P < .0001$) among the 64 HIV-1–infected subjects.

Serum opsonic activity. The opsonic activity in serum samples from 8 HIV-1–infected subjects with type-specific IgG titers of 7 for the type 3 strain was significantly higher than that in samples from 55 HIV-1–infected subjects with type-specific IgG titers of 8–10 (table 2; $P < .001$). The opsonic activity in serum samples from 56 HIV-1–infected subjects with

Table 2. Comparisons of opsonic activity against type 3 and type 9 *Streptococcus pneumoniae* in serum samples containing different levels of type-specific IgG from HIV-1–infected subjects in Uganda.

Type-specific IgG titer	Type 3 (n = 63)		Type 9 (n = 64)	
	No. of subjects	Opsonic activity, mean ± SD	No. of subjects	Opsonic activity, mean ± SD
7	8	1.15 ± 0.34[a]	1	1.1
8	28	0.88 ± 0.38	6	1.12 ± 0.67
9	21	0.99 ± 0.37	13	1.23 ± 0.54
10	6	0.82 ± 0.26	19	1.19 ± 0.55
11	...	...	17	1.13 ± 0.42
12	...	...	5	0.60 ± 0.22[b]
13	...	...	3	0.43 ± 0.15[b]

[a] $P < .001$, for subjects with IgG titers of 7 vs. subjects with IgG titers of 8–10.
[b] $P < .001$, for subjects with IgG titers of 12 and 13 vs. subjects with IgG titers of 7–11.

type-specific IgG titers of 7–11 for the type 9 strain was also significantly higher than that in serum samples from 8 HIV-1–infected subjects with type-specific IgG titers of 12 and 13 ($P < .001$). In contrast, no significant difference was found in opsonic activity against the type 3 strain in serum from 30 HIV-uninfected subjects with different titers of type 3–specific IgG ($P = .589$, by the Kruskal-Wallis test). Similarly, no significant difference in opsonic activity against the type 9 strain was found in serum from 30 HIV-uninfected subjects with different titers of type 3–specific IgG ($P = .757$).

More interestingly, a significant correlation between the peripheral blood $CD4^+$ lymphocyte count and serum opsonic activity against the type 3 strain was found for asymptomatic HIV-1–infected subjects, as shown in figure 1*A* ($r = 0.354$; $P = .043$). A similar relationship between these 2 parameters was found for the type 9 strain, although the correlation was not statistically significant (figure 1*B*; $r = 0.282$; $P = .101$). Because an inverse correlation between peripheral blood $CD4^+$ lymphocyte counts and plasma HIV-1 RNA loads already has been demonstrated in asymptomatic HIV-infected Ugandan adults [19], we compared serum opsonic activity with the plasma virus load in such subjects. We found an inverse correlation between the plasma virus load and serum opsonic activity against the type 3 strain (figure 1*C*; $r = -0.298$; $P = .092$) and the type 9 strain (figure 1*D*; $r = -0.414$; $P = .016$).

Serum opsonic activity in patients with CAP with or without HIV infection. A significant decrease in serum opsonic activity against the type 9 strain was found among 64 HIV-1–infected subjects, compared with 30 HIV-uninfected subjects (1.09 ± 0.52 vs. 1.73 ± 0.53; $P < .001$). In contrast, the levels of serum opsonic activity against the type 3 strain were slightly lower among 63 HIV-infected subjects than among 15 HIV-uninfected subjects (0.94 ± 0.37 vs. 1.04 ± 0.34; $P = .286$), although the difference was not statistically significant. Furthermore, a significant decrease in serum opsonic activity against the type 3 strain was found among HIV-1–infected patients with CAP, compared with activity levels for asymptomatic HIV-1–infected subjects (figure 2*C*), whereas no significant difference was noted in the serum levels of IgG to type 3 CPS between these 2 groups (figure 2*A*). Serum opsonic activity against the type 9 strain for HIV-infected patients with CAP was also significantly lower than that for asymptomatic HIV-infected sub-

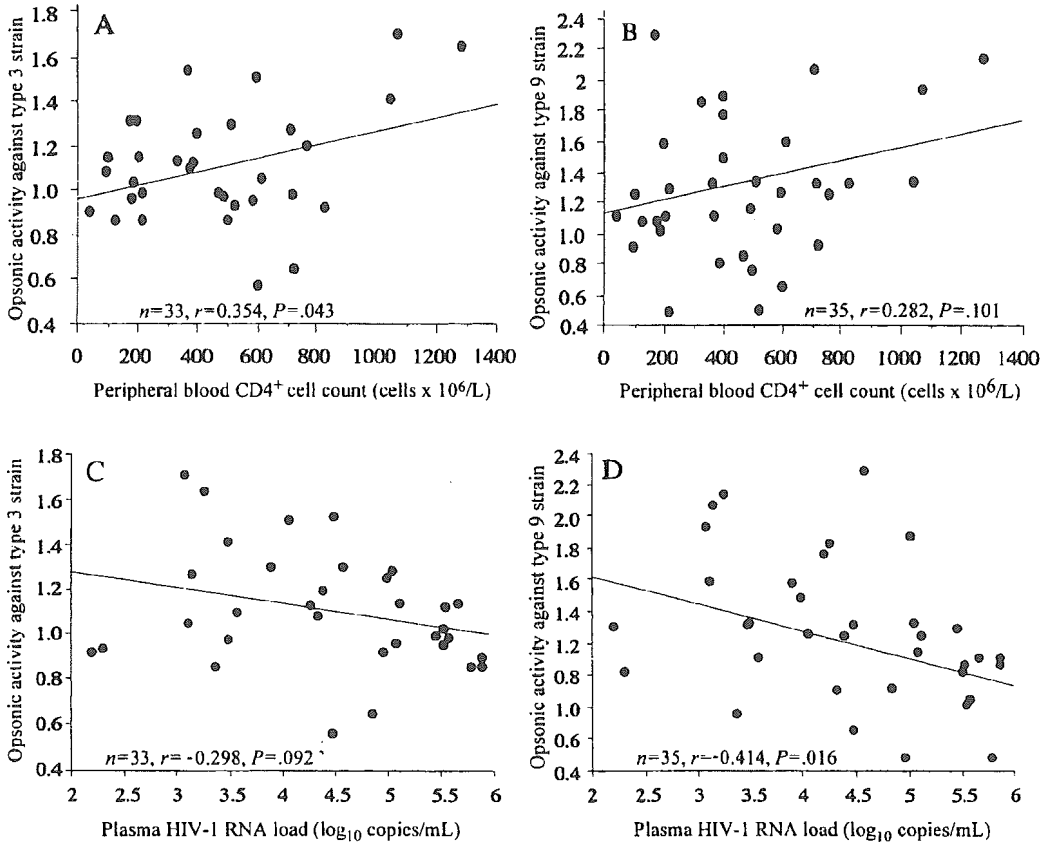

Figure 1. Correlation between peripheral blood $CD4^+$ lymphocyte count and serum opsonic activity against type 3 *(A)* and type 9 *(B) Streptococcus pneumoniae* and correlation between plasma HIV-1 RNA load and serum opsonic activity against type 3 *(C)* and type 9 *(D)* strains among HIV-1–infected Ugandan adults.

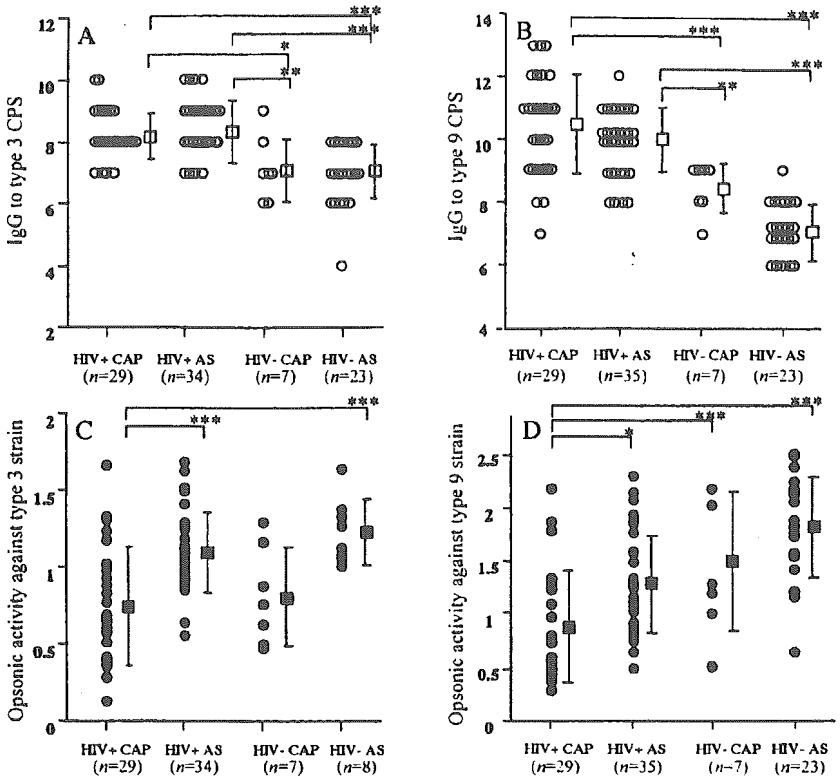

Figure 2. Comparison of levels of serum IgG to type 3 pneumococcal capsular polysaccharide (CPS) *(A)* and type 9 CPS *(B)* and serum opsonic activity against type 3 *(C)* and type 9 *(D) Streptococcus pneumoniae* among patients with community-acquired pneumonia (CAP) and asymptomatic subjects (AS) with (HIV+) or without (HIV−) HIV infection. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$, by Kruskal-Wallis test with Turkey's multiple comparison.

jects (figure 2*D*), although no significant difference was noted in the levels of serum IgG to type 9 CPS between these 2 groups (figure 2*B*). No significant differences were observed in serum opsonic activity against type 3 and type 9 strains and in serum levels of IgG to type 3 and type 9 CPS between HIV-uninfected patients with CAP and asymptomatic HIV-uninfected subjects (figure 2*C* and 2*D*).

DISCUSSION

An increase in levels of type-specific IgG in serum from HIV-infected subjects, compared with levels typical of HIV-uninfected subjects, was found. A significant correlation was also found between levels of type-specific IgG and total IgG in serum from HIV-infected subjects. In contrast, previous studies have reported that levels of serum IgG to CPS in HIV-1–infected subjects were significantly lower than those in HIV-uninfected subjects [25, 26] or that no difference was found in levels of type-specific IgG between HIV-1–infected and HIV-uninfected subjects [27].

Two studies recently reported that the avidity of type-specific IgG affects both in vitro opsonic activity and in vivo protective activity and that this response is critical in pneumococcal infection [28, 29]. These observations, however, have been largely confined to HIV-uninfected subjects. In the present study, serum samples from HIV-infected subjects that contained lower type-specific IgG titers (titers of 7 for type 3 and of 7–11 for type 9) had higher levels of opsonic activity against the type 3 and type 9 strains (table 2). These data, together with the observation of increased serum levels of type-specific IgG in HIV-infected subjects, who are susceptible to invasive pneumococcal infection, may suggest that these subjects have specific functional abnormalities of type-specific IgG. As a result, we examined the avidity of type 9–specific IgG in a limited number of serum samples from HIV-1–infected and HIV-uninfected Ugandan adults, using a method based on the dissociation of antibody-antigen complexes in the presence of 0.6 mol/L sodium thiocyanate [30]. A significant correlation between the 2 parameters was found in a group of 6 asymptomatic HIV-uninfected subjects ($r = 0.81$; $P = .04$), which is consistent with reports published elsewhere [28, 29]. In contrast, no correlation was found between the avidity levels of type-specific IgG and opsonic activity in a group of 7 asymptomatic HIV-infected subjects ($r = 0.58$; $P = .33$). These data suggest that

the function of IgG to CPS in the serum of HIV-infected subjects may be impaired.

Interestingly, we found a significant correlation between peripheral blood $CD4^+$ lymphocyte counts and serum opsonic activity against the type 3 strain among HIV-1–infected subjects (figure 1*A*). A similar relationship was found between peripheral blood $CD4^+$ lymphocyte counts and serum opsonic activity against the type 9 strain in HIV-1–infected subjects (figure 1*B*). Furthermore, the HIV-1 load correlated inversely with serum opsonic activity in asymptomatic HIV-infected subjects (figure 1*C* and 1*D*). These data suggest that HIV-1 itself may inhibit serum opsonic activity against *S. pneumoniae.* The interference of HIV with type-specific IgG and complement, therefore, will require further investigation. We also found a significantly decreased level of opsonic activity in serum from HIV-1–infected patients with CAP, compared with asymptomatic HIV-1–infected subjects (figure 2*C* and 2*D*). Because a transient increase in HIV RNA levels is found during the acute phase of bacterial pneumonia in HIV-infected adults [31], the increased virus load might suppress opsonic activity in serum from HIV-1–infected CAP patients at the time of admission. These data strongly support previous findings that indicate that the risk for bacterial pneumonia is highest among HIV-infected subjects with $CD4^+$ lymphocyte counts of <200 cells/μL in African countries, as well as industrialized countries [6, 32], and demonstrate a new finding, that a defect in immunity is a mechanism in invasive pneumococcal infections in these subjects.

A recent study reported that the use of double absorption improved the correlation between antibody concentration and opsonic activity against type 4 and type 19F pneumococcal strains [33]. The notable exceptions, however, were type 3 and type 14 CPS, in which double absorption had a minimal effect on antibody concentrations. We therefore examined whether double absorption might lead to an improvement in opsonic activity against the type 9 strain, using serum samples from 6 HIV-1–infected subjects. We found that absorption of the cross-reactive antibodies with CWPS and 22F CPS had no effect on the opsonic activity against type 9 strain, compared with absorption with CWPS alone (data not shown).

In conclusion, we report decreased levels of serum opsonic activity against *S. pneumoniae,* despite increased levels of serum IgG to type 3 or type 9 CPS, among HIV-infected Ugandan adults. Plasma HIV-1 loads correlated inversely with levels of opsonic activity, whereas peripheral blood $CD4^+$ lymphocyte counts tended to correlate directly with serum opsonic activity, against the type 3 and type 9 strains of *S. pneumoniae* among asymptomatic HIV-1–infected subjects. These data suggest that defective serum opsonic activity plays an important role in invasive pneumococcal infections in HIV-infected African adults. Further investigations will be required to clarify the specific functional abnormalities of type-specific IgG in the serum of HIV-1–infected African adults.

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References

1. Joint United Nations Programme on HIV/AIDS (UNAIDS) and the World Health Organization. AIDS epidemic update: December 2001. Geneva: UNAIDS, **2001**.
2. Gilks CF, Ojoo SA, Ojoo JC, et al. Invasive pneumococcal disease in a cohort of predominantly HIV-1 infected female sex-workers in Nairobi, Kenya. Lancet **1996**; 347:718–23.
3. Gordon SB, Chaponda M, Walsh AL, et al. Pneumococcal disease in HIV-infected Malawian adults: acute mortality and long-term survival. AIDS **2002**; 16:1409–17.
4. Jones N, Huebner R, Khoosal M, Crewe-Brown H, Klugman K. The impact of HIV on *Streptococcus pneumoniae* bacteraemia in a South African population. AIDS **1998**; 12:2177–84.
5. Karstaedt AS, Khoosal M, Crewe-Brown HH. Pneumococcal bacteremia in adults in Soweto, South Africa, during the course of a decade. Clin Infect Dis **2001**; 33:610–4.
6. Yoshimine H, Oishi K, Mubiru F, et al. Community-acquired pneumonia in Ugandan adults: short-term parenteral ampicillin therapy for bacterial pneumonia. Am J Trop Med Hyg **2001**; 64:172–7.
7. Scott JAG, Hall AJ, Muyodi C, et al. Aetiology, outcome, and risk factors for mortality among adults with acute pneumonia in Kenya. Lancet **2000**; 355:1225–30.
8. Lane HC, Masur H, Edgar LC, Whalen G, Rock AH, Fauci AS. Abnormalities of B-cell activation and immunoregulation in patients with acquired immunodeficiency syndrome. N Engl J Med **1983**; 309:453–8.
9. Shirai A, Cosentino M, Leitman-Klinman SF, Klinman DM. Human immunodeficiency virus infection induces both polyclonal and virus-specific B cell activation. J Clin Invest **1992**; 89:561–6.
10. Morris L, Binley JM, Clas BA, et al. HIV-1 antigen–specific and –non-specific B cell responses are sensitive to combination anti-retroviral therapy. J Exp Med **1998**; 188:233–45.
11. Notermans DW, Jong'JD, Goudsmit JAAP, et al. Potent antiretroviral therapy initiates normalization of hypergammaglobulinemia and a decline in HIV type 1–specific antibody responses. AIDS Res Hum Retroviruses **2001**; 17:1003–8.
12. Knapp S, Leemans JC, Florquin S, et al. Alveolar macrophages that have a protective anti-inflammatory role during murine pneumococcal pneumonia. Am J Respir Crit Care Med **2003**; 167:171–9.
13. Musher DM, Chapman AJ, Goree A, Jonsson S, Briles D, Baughn RE. Natural and vaccine-related immunity to *Streptococcus pneumoniae.* J Infect Dis **1986**; 154:245–56.
14. Ellis M, Gupta S, Galant S, et al. Impaired neutrophil function in patients with AIDS or AIDS-related complex: a comprehensive evaluation. J Infect Dis **1988**; 158:1268–78.
15. Murphy PM, Lane HC, Fauci AS, Gallin JI. Impairment of neutrophil bactericidal capacity in patients with AIDS. J Infect Dis **1988**; 158: 627–30.
16. Flo RW, Naess A, Nilsen A, Harthug S, Solberg CO. A longitudinal study of phagocyte function in HIV-infected patients. AIDS **1994**; 8: 771–7.
17. Mundy LM, Auwaerter PG, Olach D, et al. Community-acquired pneumonia: impact of immune status. Am J Respir Crit Care Med **1995**; 152:1309–15.
18. Saito A, Miki F, Oizumi K, et al. Clinical evaluation methods for new antimicrobial agents to treat respiratory infections: report of the Com-

mittee for the Respiratory System, Japan Society of Chemotherapy. J Infect Chemother **1999**; 5:110–23.
19. Oishi K, Hayano M, Yoshimine H, et al. Expression of chemokine receptors on $CD4^+$ T cells in peripheral blood from HIV-infected individuals in Uganda. J Interferon Cytokine Res **2000**; 20:597–602.
20. Vitharsson G, Jonsdottir I, Jonsson S, Valdimarsson H. Opsonization and antibodies to capsular and cell wall polysaccharides of *Streptococcus pneumoniae*. J Infect Dis **1994**; 170:592–9.
21. Kurono Y, Yamamoto M, Fujihashi K, et al. Nasal immunization induces *Haemophilus influenzae*–specific Th1 and Th2 response with mucosal IgA and systemic IgG antibodies for protective immunity. J Infect Dis **1999**; 180:122–32.
22. Tsuda H, Yamashita Y, Toyoshima K, et al. Role of serotype-specific polysaccharide in the resistance of *Streptococcus mutans* to phagocytosis of human polymorphonuclear leukocytes. Infect Immun **2000**; 68: 644–50.
23. Oishi K, Koles NL, Guelde G, Pollack M. Antibacterial and protective properties of monoclonal antibodies reactive with *Escherichia coli* O111: B4 lipopolysaccharide: relation to antibody isotype and complement-fixing activity. J Infect Dis **1992**; 165:34–45.
24. Fantin B, Joly V, Elbim C, et al. Lymphocyte subset counts during the course of community-acquired pneumonia: evolution according to age, human immunodeficiency virus status, and etiologic microorganisms. Clin Infect Dis **1996**; 22:1096–8.
25. Moja PH, Jalil A, Quesnel M, et al. Humoral immune response within the lung in HIV-1 infection. Clin Exp Immunol **1997**; 110:341–8.
26. Janoff EN, Fasching C, Ojoo JC, O'Brien J, Gilks CF. Responsiveness of human immunodeficiency virus type 1–infected Kenyan woman with or without prior pneumococcal disease to pneumococcal vaccine. J Infect Dis **1997**; 175:975–8.
27. French N, Gilks CF, Mujugira A, Fasching C, O'Brien J, Janoff EN. Pneumococcal vaccination in HIV-infected adults in Uganda: humoral response and two vaccine failures. AIDS **1998**; 12:1683–9.
28. Usinger WR, Lucas AH. Avidity as a determinant of the protective efficacy of human antibodies to pneumococcal capsular polysaccharides. Infect Immun **1999**; 67:2366–70.
29. Musher DM, Phan HM, Watson DA, Baughn RE. Antibody to capsular polysaccharide of *Streptococcus pneumoniae* at the time of hospital admission for pneumococcal pneumonia [erratum: J Infect Dis 2000; 182:1011]. J Infect Dis **2000**; 182:158–67.
30. Anttila M, Voutilainen M, Jantti V, Eskola J, Kayhty H. Contribution of serotype-specific IgG concentration, IgG subclass and relative antibody avidity to opsonophagocytic activity against *Streptococcus pneumoniae*. Clin Exp Immunol **1999**; 118:402–7.
31. Bush CE, Donovan RM, Markowitz NP, et al. A study of HIV RNA viral load in AIDS patients with bacterial pneumonia. J Acquir Immune Defic Syndr Hum Retrovirol **1996**; 13:23–6.
32. Hirschtick RE, Glassroth J, Jordan MC, et al. Bacterial pneumonia in persons infected with the human immunodeficiency virus. Pulmonary Complications in HIV Infection Study Group. N Engl J Med **1995**; 333:845–51.
33. Concepcion NF, Frasch CE. Pneumococcal type 22F polysaccharide absorption improves the specificity of a pneumococcal-polysaccharide enzyme-linked immunosorbent assay. Clin Diagn Lab Immunol **2001**; 8:266–72.

特集　肺炎：診断と治療の進歩

トピックス

I. 日常診療においてよくみられる肺炎
1. 細菌性肺炎（肺炎球菌性肺炎を中心に）

大石　和徳　　吉嶺　裕之

要　旨

肺炎球菌性肺炎の成立機序として，呼吸器ウイルスの先行感染による気道上皮細胞への菌付着の重要性が示唆される．本邦の114症例の成人における肺炎球菌性肺炎症例の臨床像と起炎菌の薬剤耐性を検討した．患者の平均年齢は67.4歳で，菌血症頻度（2.6%），致命率（4.4%）は欧米の成績に比較して低率であった．起炎菌のペニシリンおよびマクロライド耐性は高いものの，重症度および致命率と薬剤耐性との関連は明らかでなかった．〔日内会誌　94：2256～2260，2005〕

Key words：肺炎球菌，気道上皮付着，薬剤耐性，マクロライド併用療法

はじめに

肺炎は本邦における死因の第四位であり，2003年には約９万５千人が肺炎で死亡している．細菌性肺炎は多様な呼吸器病原性菌により惹起される肺実質感染症であり，市中肺炎のうちの大部分を占めている．細菌性肺炎の病因は市中肺炎と院内肺炎とで大きく異なるが，市中肺炎の起炎菌としては肺炎球菌が最も頻度が高く，市中肺炎の約20～40%を占めている．肺炎球菌に続いて，インフルエンザ菌，黄色ブドウ球菌，モラキセラ・カタラーリス，肺炎桿菌，ストレプトコッカス・ミレーリーグループ，嫌気性菌等がこれらに続く．本稿では肺炎球菌性肺炎について診断，治療の現状とその問題点について記載する．

おおいし　かずのり，よしみね　ひろゆき：長崎大学熱帯医学研究所感染症予防治療分野

1. 細菌性肺炎の成立機序

ライノウイルスやインフルエンザをはじめとするウイルス性上気道炎による気道障害は病原性菌の上気道への付着を助長し，その後の二次性細菌性肺炎を惹起する．これまでに，肺炎球菌は菌体表面のphosphorylcholine（PC）を介して，細胞表面上のplatelet activating factor（PAF）リセプターに結合し，細胞内に侵入することが知られている．その後，このような肺炎球菌の宿主細胞への付着・侵入機序とウイルス感染のかかわり合いから，新たな肺炎成立機序が明らかになっている．すなわち，ライノウイルスを*in vitro*で感染させた気道上皮細胞にはPAFレセプター発現が増強し，肺炎球菌の付着が亢進することが示されている[1]．また，インフルエンザウイルスを*in vitro*で肺上皮細胞に感染させた場合に，肺炎球菌の付着能が亢進することも報告されている．この実験系にノイラミニダーゼ阻害剤であるoseltamivirを添加すると菌付着が低下することから，インフルエンザウイルスのノ

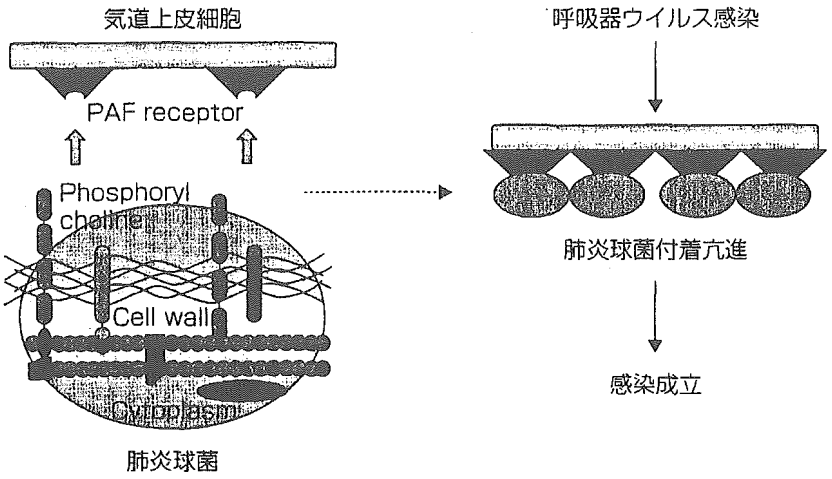

図1. 肺炎球菌呼吸器感染症の成立機序
呼吸器ウイルス感染に伴う気道上皮細胞表面上のPAFレセプター発現が増加し，肺炎球菌の気道上皮付着が亢進する

イラミニダーゼは気道傷害を介して肺炎球菌付着に関与することが示されている．さらに，マウス肺炎実験モデルにおいてもインフルエンザウイルス先行感染において肺炎球菌性肺炎が重症化することも明らかになっている．

一方，Madhiらは南アフリカの乳幼児を対象とした研究において，ウイルス関連肺炎に肺炎球菌が重要な役割を果たすことを示唆している[2]．すなわち，乳幼児の肺炎球菌性肺炎の診断における血液培養の感度は低く，他に感度の高い診断法は無い．一方，HIV感染の無い乳幼児に対しては肺炎球菌コンジュゲートワクチン接種がその侵襲性肺炎球菌性感染症を85～97%抑制する事実から，このワクチン効果がウイルス肺炎における肺炎球菌の役割を明らかにするための感度の高いプローブとなると考えられる．この著者らは乳幼児のワクチン接種群（n＝18,245）におけるウイルス関連肺炎の発症頻度をプラセボ群（n＝18,268）と比較し，コンジュゲートワクチンはインフルエンザA，RSV，パラインフルエンザウイルスの検出された肺炎の発症を22～45%も抑制したとしている．この結果は，肺炎球菌がウイルス関連肺炎の発症に重要な役割を果たすこと，またこれらのウイルスが細菌性肺炎の発症に関与していることを示唆している．

同様にインフルエンザ菌と気道上皮の付着・侵入機構にもPAFレセプターが関与する事が報告されている．この事実から，肺炎球菌と同様に先行するウイルス感染がインフルエンザ菌感染の頻度を増加させることが推察される．

上述の報告を要約すると，呼吸器ウイルスは気道上皮細胞を傷害し，レセプター発現を亢進することで，肺炎球菌の付着を増加させ，感染増悪のリスクを高めていることが示唆される（図1）．

2. 診断の進歩：肺炎球菌尿中抗原

最近，肺炎球菌尿中抗原検査（Binax NOW *Streptococcus pneumoniae*）が保険適応になり，その臨床応用が進んでいる[3]．この検査法は，肺炎球菌感染症患者の血中抗原が尿中に濃縮され，尿中抗原陽性になることを利用して開発された．尿検体は採取が容易であり，気道分泌物等のように口腔内細菌による汚染の可能性も無い．従って，肺炎球菌尿中抗原検査は肺炎球菌による肺炎，菌血症，髄膜炎等の簡便な細菌学的補助診断として意義がある．

1）肺炎球菌尿中抗原検査の原理

本キットはimmunochromatographic membrane assayの原理を利用した，肺炎患者の尿中および髄膜炎患者の髄液中の肺炎球菌莢膜共通多糖抗原（C-polysaccharide）の迅速検出法である．

2）臨床成績

51例の肺炎球菌性肺炎症例における肺炎球菌尿中抗原検出において，菌血症を伴う28例，菌血症を伴わない23例における尿中抗原陽性率はそれぞれ82.1%と78.3%といずれも高率で，両者間の差は認められなかった．全体の尿中抗原検査の感度は80.4%で特異度は97.2%であった．これらの結果は，菌血症の存在にかかわらず，肺炎球菌性肺炎における尿中抗原は，とりわけ菌血症を伴わない肺炎球菌性肺炎の診断に有用であることを示唆している．さらに英国における肺炎球菌性菌血症107例を対象とした検討においても，尿中抗原検査の感度は82%，特異度は97%であった．また，尿中抗原は治療7日後でも80～90%が陽性であった．

3）肺炎球菌抗原検査の問題点

小児における鼻咽頭への肺炎球菌の定着が尿中抗原結果に影響することが報告されている．鼻咽頭に肺炎球菌を保有している小児（66.6%）の尿中抗原陽性率は明らかに肺炎球菌を保有しない小児の陽性率（32.9%）より高かったと報告されている．また，肺炎球菌を保有していない小児において尿中抗原陽性率が高い理由として，低いレベルの肺炎球菌定着あるいはC-polysaccharideを保有する*Streptococcus mitis*の定着による可能性が考えられている．

さらに，肺炎発症後の患者において数週間も尿中抗原が陽性になることも報告されており，尿中抗原陽性の判定には肺炎球菌肺炎既往の有無に十分留意する必要がある．

3．本邦の肺炎球菌性肺炎の実態調査

1）肺炎球菌性市中肺炎の特徴（表1）

近年，本邦をはじめとする東アジア諸国において，肺炎球菌のβラクタム耐性やマクロライド耐性の頻度が高まり，その抗菌薬治療上の問題点が指摘されている．今回，我々は全国20施設で肺炎球菌性市中肺炎の臨床像，起炎菌の薬剤耐性，血清型の実態を調査した．研究実施期間は2001～2003年で，通常の方法で肺炎を診断し，細菌学的には喀痰および血液培養を実施して起炎菌を決定した．114例において，肺炎球菌は109例が喀痰，3例が血液，1例が胸水，1例が気管支肺胞洗浄液から分離され，菌血症を伴う肺炎球菌性肺炎の頻度は欧米に比較して低かった．また，114例中，89例（78.1%）は入院し，残りの25例は外来で治療された．患者の平均年齢は67.4歳（20～99歳）で，男性が59.6%を占めていた．全症例の71.9%に基礎疾患が認められ，その内訳は慢性呼吸器疾患（39.5%），糖尿病（12.3%），脳血管障害（8.8%）などであった．日本呼吸器学会のガイドラインに従った重症度分類では，重症33.3%，中等症42.1%，軽症24.6%であった．入院患者の平均在院日数は軽症でも15.9日，中等症24.1日，重症34.3日と，欧米に比較して明らかに在院日数は長かった（図2）．これらの114症例中，109例（95.6%）は治療により軽快したものの，5例（4.4%）が死亡した．この致命率4.4%は欧米の成績に比較すると低率であった．図3には入院症例のうち治癒例（n＝77）と死亡例（n＝5）の入院日数を示した．死亡例では入院日数が1週間未満であり，急速な経過を示した事が伺える．

2）肺炎球菌の薬剤感受性と耐性遺伝子

肺炎球菌114株のペニシリン感受性成績では，26株（22.8%）がペニシリン耐性であり（MIC 2μg/mlが25株，4μg/mlが1株），ペニシリン非感受性（MIC＞0.12μg/ml）以上は66株（57.9%）

表1. 肺炎球菌性市中肺炎114症例の臨床像の特徴

平均年齢	67.4歳（男性59.6%）
菌血症陽性例	2.6%
基礎疾患合併	71.9%
慢性呼吸器疾患合併	39.5%
重症例の頻度	33.3%
致命率	4.4%

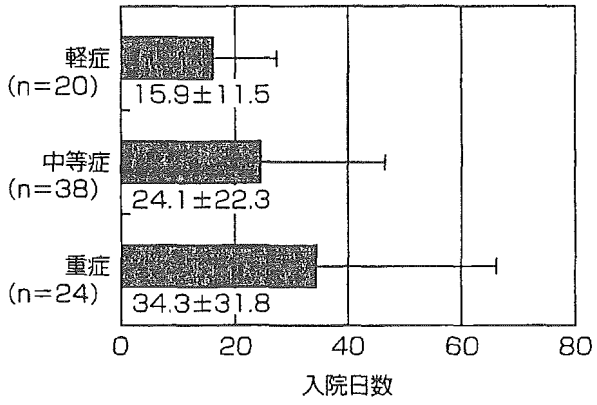

図2. 肺炎球菌性肺炎患者の入院日数
軽症，中等症，重症例の順に入院日数は延長している．軽症でも入院日数は2週間を超えている．

であった．肺炎球菌のβラクタム耐性を担っている*pbp*遺伝子変異の検討では，42株（36.8%）が*pbp1a*＋*2x*＋*2b*遺伝子変異を有し（genotypic PRSP），28.1%が*pbp2x*遺伝子変異を有していた．*pbp1a*＋*2x*＋*2b*遺伝子変異株のMICは0.25～4.0μg/ml，MIC50が2μg/mlとペニシリン耐性を示すのに対し，*pbp2x*遺伝子変異株はMIC範囲0.03～0.13μg/ml，MIC50が0.03μg/mlとペニシリン感受性であった．*pbp*遺伝子変異を認めなかったのは13株（11.4%）のみに過ぎなかった．

一方，肺炎球菌114株のマクロライド耐性に関与する*erm* B遺伝子，*mef* A遺伝子の頻度についても検討した．*erm* B遺伝子は23S rRNA methylaseのmetylationをコードし，*mef* A遺伝子はマクロライドのefflux に関与するとされている．結果として，114株中*erm* B遺伝子保有株（50株：56.1%）が最も多く，続いて*mef* A遺伝子保有株（26株：22.8%），遺伝子非保有株（24株：21.1%），

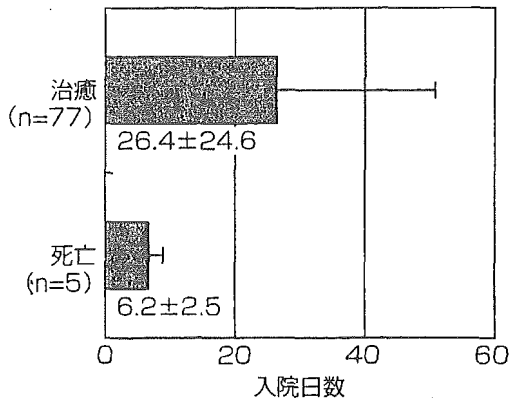

図3. 肺炎球菌性肺炎患者の入院日数
死亡例の入院日数は1週間以内と短かく，死亡症例の経過が早い．

mef A＋*erm* B遺伝子保有株（7株：6.1%）の順であった．*erm* B遺伝子保有株は*mef* A遺伝子保有株に比較してエリスロマイシン耐性が顕著（MIC：0.5～128μg/ml，MIC50＝128μg/ml）であった．

3）肺炎球菌性肺炎の重症度および致命率と薬剤耐性

114症例の肺炎球菌肺炎の重症度と起炎菌の薬剤耐性遺伝子保有との関連性について検討した．ペニシリン感受性株である*pbp2x*株は重症の傾向を示し，*pbp1a*＋*2x*＋*2b*株はより軽症の傾向が認められた．しかしながら，重症度および致命率とペニシリン耐性遺伝子の分布に明らかな有意差は認められなかった．同様に，重症度および致命率とマクロライド耐性遺伝子分布との関連性も認められなかった．

4. 治療の問題点と最近の動向

これまでに，Metlayらは成人の菌血症を伴う肺炎球菌性肺炎患者において，ペニシリン非感受性肺炎球菌がその院内死亡のリスク因子になると報告している[4]．しかしながら，我々の成績では菌血症を伴わない肺炎球菌性肺炎が大半で，致命率も4.4%と低率であったこともあり，死亡と肺炎球菌のペニシリン耐性との間には明らか

な関係は認められていない．これまでの多くの研究においても，肺炎球菌性肺炎の死亡とペニシリン耐性の相関は明らかでない．さらには，最近の論文は不適切なβラクタム剤による治療であっても結果的に肺炎の致命率を上昇させていないとしている．一方，肺炎球菌性肺炎のマクロライド耐性と治療失敗が相関するとした報告がある．当然ながら，肺炎球菌のマクロライド耐性が高い地域では市中肺炎患者のマクロライド単剤治療には注意が喚起されている．

このように，現時点の肺炎球菌性肺炎の治療において，起炎菌の薬剤耐性に伴う治療失敗はある．しかしながら，適切な治療の有無にかかわらず，致命率の増加を招く事態は起こっていないと言えよう．

近年，海外では肺炎球菌肺炎に対するβラクタム剤とマクロライド剤の有用性が注目されている．Martinezらは，過去10年間の菌血症を伴う409症例の肺炎球菌性肺炎のβラクタム剤ベースのエンペリックな治療を評価し，マクロライド系薬の併用が肺炎死亡のリスクを低下させると報告している[5]．この結果は，起炎菌未定の，入院が必要な市中肺炎の治療にβラクタム剤とマクロライド系薬併用の推奨を支持している．さらに，この結果は他のレトロスペクティブ研究により確認されており，今後プロスペクティブ研究により重症肺炎に対するβラクタム剤とマクロライド系薬併用療法の評価が早急に求められている．

文　献

1) Ishizaka S, et al: Effects of rhinovirus infection on the adherence of *Streptococcus pneumoniae* to cultured human airway epithelial cells. J Infect Dis 188: 1928–1939, 2003.
2) Madhi SA, et al: A role for *Streptococcus pneumoniae* in virus-associated pneumonia. Nat Med 10: 811–813, 2004.
3) 陳　蒙，大石和徳：肺炎球菌尿中抗原検出法．日本臨床 63: 159–162, 2005.
4) Metlay JP, et al: Impact of penicillin susceptibility on medical outcome for adult patients with bacteremic pneumococcal pneumonia. Clin Infect Dis 30: 520–528, 2000.
5) Martinez JA, et al: Addition of a macrolide to β-lactam-based empirical antibiotic regimen is associated with lower in-hospital mortality for patients with bacteremic pneumococcal pneumonia. Clin Infect Dis 36: 389–395, 2003.

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Cloning of the varicella-zoster virus genome as an infectious bacterial artificial chromosome in *Escherichia coli*

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Abstract

The complete genome of the varicella-zoster virus (VZV) Oka strain has been cloned as a bacterial artificial chromosome (BAC). Following electroporation into *Escherichia coli* (*E. coli*) strain DH10B, the VZV BAC was stably propagated over multiple generations of its host. Human embryonic lung (HEL) cells transfected with VZV BAC DNA recovered from DH10B showed cytopathic effect (CPE), and virus spread to neighbouring cells was observed. BAC vector sequences are flanked by *lox*P sites and, coinfection of the reconstituted virus, with a recombinant adenovirus expressing Cre recombinase removed the bacterial sequences. The resulting recombinant rV02 grew as well as the parental virus in HEL cells. The recombinant VZV will promote VZV research and increase use of the viral genome as an investigative tool.

Keywords: VZV; BAC; Recombinant virus

1. Introduction

Varicella-zoster virus (VZV) is a human herpesvirus that causes chickenpox (varicella) and shingles (herpes zoster). A live attenuated varicella vaccine (Oka strain) was originally developed by Takahashi et al. [1] and is routinely used in children in Japan and other countries, including the United States of America. We previously determined the DNA sequence of the Oka varicella vaccine virus (V-Oka) and its parental virus (P-Oka) [2]. That study provided critical information for investigating the genes involved in VZV attenuation, but the mechanism of attenuation is still not completely clear. Recently, the genomes of a number of herpesviruses were cloned into a bacterial artificial chromosome (BAC) [3–20]. This bacterial genetics method allows the stable maintenance of the viral genome in *Escherichia coli* and the introduction of mutations into the genome [21]. In this report, we describe the construction of an infectious BAC clone for VZV, which contains the complete genome of P-Oka, in *E. coli*. After the transfection of the VZV BAC DNA into human embryonic lung (HEL) cells, infectious viruses were recovered. Because the BAC vector sequence is flanked by *lox*P sites, it is excisable from the genome of the reconstituted virus by Cre recombinase, generating a recombinant virus without the BAC vector sequence. The in vitro replication characteristics of the recombinant VZV were indistinguishable from those of the parental P-Oka virus.

2. Materials and methods

2.1. Cells and viruses

P-Oka was isolated in Japan from a patient with varicella and was propagated only in human embryonic lung cells [2].

2.2. Preparation of viral and BAC DNA

To isolate the VZV DNA from infected cells, the infected cells were treated with 0.05% EDTA/PBS (−) and collected by centrifugation at 500 × *g*. The cells were lysed in a solu-

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Table 1
Primers used for generation of recombinant plasmid

Primer name	Sequence	Product (size in bp)
VZ11F	*5′-TATA* ACTAGT$_{SpeI}$ GCGGCCGC$_{NotI}$ TTACGAAAACGTGCATG-3′	VZ ORF11(2652)
VZ11R	*5′-CGCG* ACCTGGT$_{SexAI}$ TTTTACAAACTCCTTTGTGG-3′	
VZ12F	*5′-GCGC* ACCAGGT$_{SexAI}$ CTCTGTTTAGACCTTAAAATTTG-3′	VZ ORF12(2164)
VZ12R	*5′-TATA* GCGGCCGC$_{NotI}$ TTTTAATCTGGTTGTGGAAATG-3′	

Restriction enzyme sites are underlined in the oligonucleotide sequence, while sequence in italic letters indicate additional bases that are not present in the VZV sequences.

tion containing 50 mM Tris–HCl (pH 7.6), 3.6 mM $CaCl_2$, 5 mM $MgSO_4$, 125 mM KCl, 0.5mM EDTA, 0.5% NP40, and 0.5% sodium deoxycholate. The lysates were spun at 80,000 × *g* for 2 h at 4 °C through a 7– 40% glycerol gradient in an SW28 rotor (Beckman). The pellet was treated with buffer (10 mM Tris–HCl, 150 mM NaCl, 10 mM EDTA, 0.5% SDS) containing proteinase K (500 μg/ml). The viral DNA was extracted with phenol–chloroform and precipitated with isopropanol. Circular viral DNA was isolated from infected cells by the Hirt extraction procedure [22]. VZV BAC DNA was isolated using a NucleoBond PC 100 kit (Macherey-Nagel) following the manufacturer's protocol.

2.3. Plasmid construction

The plasmid pHA-2 containing guanine phosphoribosyl transferase gene (gpt), gfp, *lox*P and BAC sequences was used in this study [3]. DNA fragments encoding ORF 11 and ORF 12 of P-Oka were amplified from VZV genomic DNA by PCR. The primers used for the PCR amplification of the targeting regions are shown in Table 1. Following amplification, the ORF 11 PCR product was digested with *Spe*I and *Sex*AI, and the ORF 12 PCR product was digested with *Not*I and *Sex*AI. Both fragments were inserted into pBluescript SK (Stratagene), which had been digested with *Spe*I and *Not*I, generating the resultant plasmid SK/VZ11–12. Next, the BAC along with the gpt and gfp gene and flanking loxP sites was released from pHA-2 by digestion with *Pac* I, and the DNA ends were blunt-ended by treatment with T4 DNA polymerase. The resulting fragment was inserted into the blunt-ended *Sex*AI site of SK/VZ11–12. The BAC vector (named pKSO-gpt) [10] along with gfp and flanking loxP sites is contained in plasmid pHA2. Following transformation into *E. coli* strain DH10B, the transformants were plated on agar containing 17 μg/ml chloramphenicol. The resulting plasmid was called pHA-2/VZV11–12 (Fig. 1C).

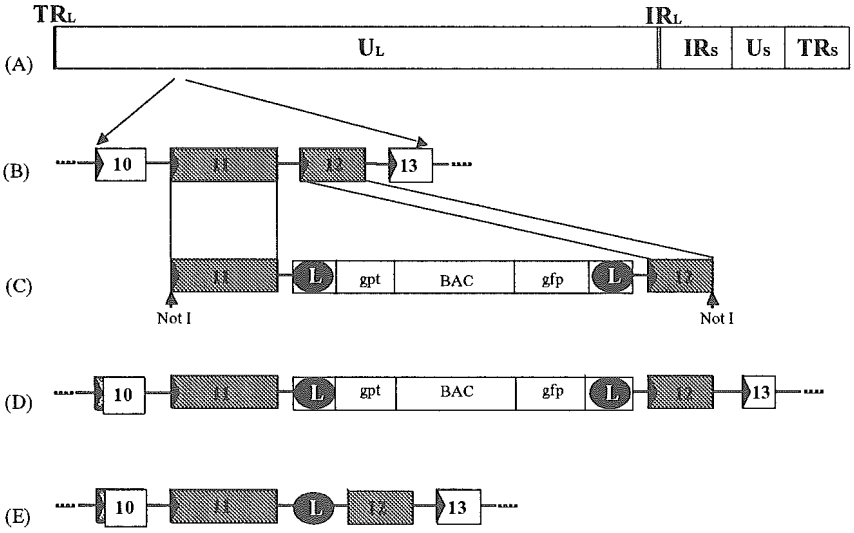

Fig. 1. Construction of the VZV genome BAC in *E. coli*. The VZV genome (A) is about 125-kbp long and consists of terminal repeat (TR), unique long (UL), internal repeat (IR), and unique short (US) DNA domains. The recombinant plasmid, PHA-2/VZV11–12 (C) contained 2.0 kbp each of two sequences from VZV (shaded boxes, 11 and 12) as well as the BAC vector, the gpt gene, and the gfp gene, flanked by *lox*P sites. The ORF11 and the ORF12 fragments of the genome of the VZV wild-type strain, P-Oka (shaded boxes, 11 and 12) were generated by PCR amplification using the appropriate primers (Table 1). The recombinant VZV BAC clone was generated in HEL cells by homologous recombination of the recombinant plasmid PHA-2/VZV11–12 and the wild-type VZV virus, P-Oka. Electroporation of the circular VZV BAC cloned genome into *E. coli* generated the VZV BAC plasmid. The recombinant VZV, rV01 (D) was generated by transfection of the VZV BAC plasmid into HEL cells. The recombinant VZV, rV02, which lacked the BAC vector sequences (E), was generated by superinfection of VZV rV01 and a recombinant adenovirus, AxCANCre. The VZV, rV02 contains a single *lox*P site. The circle enclosing an L represents the *lox*P site.

2.4. Generation of the VZV BAC

The recombinant plasmid pHA-2/VZ11–12 (Fig. 1C) containing the BAC sequence and flanking homologies to the VZV genes ORF-11 and -12 was linearized by *Not*I treatment. Linearized pHA-2/VZ11–12 (0.2 μg) was transferred into HEL cells by electroporation with a Nucleofection unit (Amaxa). One day posttransfection, the transfected cells were overlaid with the harvested HEL cells that had been infected with P-Oka, and had shown a cytopathic effect (CPE) in more than 80% of the cells. Two days later, the overlaid HEL cells were harvested. To enrich for the recombinant viruses, the cells, which contained pHA-2/VZ11–12 DNA and infected with P-Oka viruses, were harvested and transferred to a new flask of subconfluent HEL cells, and 50 μM mycophenolic acid and 200 μM xanthine were then added to select for recombinant virus using the gpt marker. Further enrichment for recombinant viruses was done by both drug selection and limiting dilution in 96-well plates. After several rounds of selection, circular viral DNAs were isolated from infected cells and transferred into *E. coli* DH10B (Invitrogen) by electroporation using a Bio-Rad *E. coli* Pulser with 0.2-cm cuvettes at 2.5 kV [23]. The transformants were plated on agar containing 17 μg/ml chloramphenicol.

2.5. Reconstitution of infectious virus

To produce the recombinant virus rV01 from VZV BAC, 1 μg of VZV-BAC DNA was transferred into HEL cells by electroporation using a Nucleofection unit (Amaxa). After the electroporation, the cells were propagated in six-well dishes for 3–4 days. When the electroporated cells became confluent in each well of dish, the cells were then propagated in a new flask, and observed under a microscope until a typical cytopathic effect with green fluorescence appeared. Typical CPEs were observed in the cells at 1–2 days after the cells were spread. To remove the BAC vector sequence, HEL cells were infected with a recombinant adenovirus, AxCANCre, which expresses the Cre recombinase (kindly provided by Dr. Yasushi Kawaguchi, Nagoya University School of Medicine) [17] at a multiplicity of 100 PFU per cell. After 2 h of viral adsorption, the cells were washed with PBS (−) and cultured with Dulbecco's minimal essential medium (DMEM) containing 5% fetal calf serum (FCS). At 24-h post infection (PI) with the recombinant adenovirus, the cells were superinfected cell–cell with the VZV BAC virus, rV01, and the infection of VZV was allowed to proceed until a typical CPE without green fluorescence appeared.

3. Results and discussion

3.1. Cloning of the VZV genome in E. coli

The plasmid pHA-2, which contains BAC sequences, guanine phosphoribosyl transferase (gpt) [24] as the selection marker, the green fluorescence protein (gfp) gene, flanked by *lox*P sites, was used in this study [3]. For construction of the VZV BAC, the region between open reading frame (ORF) 11 and ORF12 of P-Oka was chosen for integration of the BAC vector (Fig. 1B), because this region is predicted to be nonessential for replication based on herpes simplex virus type 1 (HSV-1), which belongs to the same subfamily and has similar characteristics [25]. The recombinant plasmid pHA-2/VZ11–12 (Fig. 1C) linearized by *Not*I treatment was introduced into HEL cells by electroporation. The transfected HEL cells were overlaid with P-Oka infected HEL cells, showing a cytopathic effect (CPE). To enrich for the recombinant viruses, the cells, which contained pHA-2/VZ11–12 DNA and P-Oka viruses, were transferred to a newly prepared flask of subconfluent HEL cells, and typical plaques of VZV exhibiting green fluorescence under a fluorescence microscope were seen in the cells, indicating that the recombinant virus had replicated in the cells, because the gfp in the recombinant virus was expressed. Recombinant viruses (Fig. 1D) were selected using mycophenolic acid and xanthine by utilizing the gpt marker. After several rounds of selection, circular viral DNAs were isolated from infected cells and transferred into *E. coli* by electroporation. Restriction enzyme analysis of DNA isolated from single *E. coli* colonies allowed us to identify a bacterial clone containing a BAC with a full-length VZV genome (data not shown).

3.2. Stability of the VZV BAC plasmid VZV-BAC in E. coli

BAC plasmids are usually propagated in the *E. coli* strain DH10B, which carries a *rec*A mutation to minimize the propensity for recombination [3]. Several herpesvirus-BACs can be stably maintained in this *E. coli* strain [3–20]. The MHV-68 genome that contains a large number of repeated sequences, showed some heterogeneity with regard to the number of repeated sequences when cloned and propagated in *E. coli* [3]. However, the loss of repeated sequences does not appear to influence the reconstitution or in vitro growth properties of the infectious virus [3]. Therefore, we examined the stability of VZV-BAC in *E. coli*. Bacteria containing original VZV-BAC were grown for three passages of 24 h each and finally plated on agar containing 17 μg/ml chloramphenicol. Overnight cultures were grown from single colonies on agar plate, and the DNA was isolated from these cultures and analyzed by restriction enzyme digestion and gel electrophoresis (Fig. 2, lanes 1–5). All five colonies shown in Fig. 2 showed a *Bam*HI restriction pattern that was identical to that of the original VZV-BAC (Fig. 2, lane 6), indicating a high stability of the VZV BAC in *E. coli*. Furthermore, we confirmed the integration of the BAC DNA sequences between ORF 11 and ORF12 in the VZV genome by sequencing (data not shown).

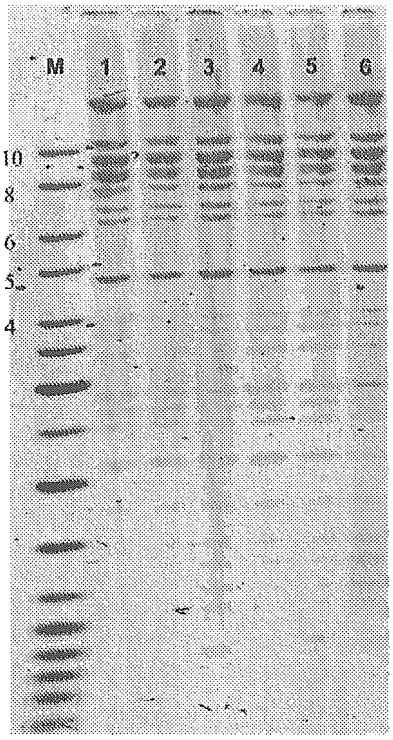

Fig. 2. Stability of the VZV BAC DNA in *E. coli*. The original VZV BAC clone was propagated three times in *E. coli* DH10B. Afterwards, bacteria containing VZV-BAC were plated on agar containing chloramphenicol. DNA was isolated from single colonies, digested with *Bam*HI, and analyzed by gel electrophoresis on 0.5% agarose gels in 0.5× Tris-borate–EDTA buffer for 24 h at 2.5 V/cm. DNA fragments were visualized by ethidium bromide staining. All five clones (lanes 1–5) showed a restriction enzyme digestion pattern that was identical to that of the original VZV BAC DNA (lane 6). Molecular sizes in (kilobase pairs) are shown on the left. The picture of the agarose gel electrophoresis was taken with a Bio-Rad Molecular Imager FX (Bio-Rad).

3.3. Reconstitution of infectious virus from the VZV BAC DNA

To produce the recombinant virus from VZV BAC DNA, VZV-BAC DNA was transfected into HEL cells by electroporation. Several days later, typical CPEs were observed in the cells. The resulting recombinant VZV containing the BAC DNA sequence (Fig. 1D) was named rV01. Since herpesviruses containing the additional BAC may have an altered phenotype due to the insertion of non-viral sequences, we excised the BAC vector sequences from rV01. The BAC vector sequences, including the gpt and gfp genes, were excised by site-specific recombination using the flanking by *lox*P sites. This was achieved by co-infection of HEL cells with the recombinant adenovirus, AxCANCre, which expresses the Cre recombinase [17] [26] and rV01. Following this, a typical VZV CPE without green fluorescence appeared. Correct excision of BAC sequences from the recombinant virus rVo2 (Fig. 1E), was confirmed by sequencing (data not shown).

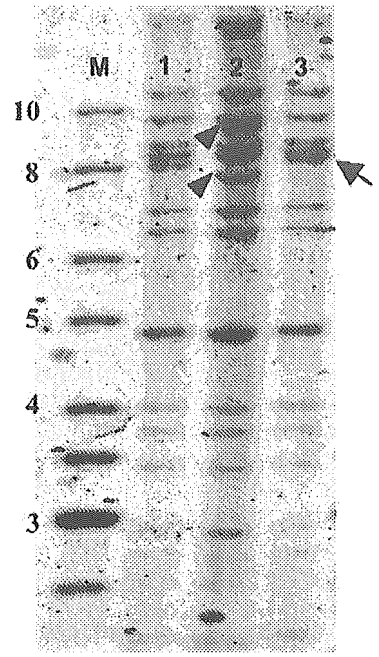

Fig. 3. Structural analysis by *Bam*HI-digestion of the VZV BAC plasmid and reconstituted viral genome. Wild-type VZV DNA (P-Oka) isolated from infected cells (lane 1), VZV BAC DNA isolated from *E. coli* (lane 2), and reconstituted virus rV02 DNA (without the BAC vector sequences) isolated from infected cells (lane 3) were digested with *Bam*HI and separated by gel electrophoresis. Fragments in the VZV BAC DNA(lane 2) appearing as a consequence of the inserted BAC sequences are marked by arrowheads. The fragment in the reconstituted virus rV02 DNA (lane 3) caused by the remaining *lox*P sequence is marked by an arrow. Molecular sizes (in kilobase pairs) are shown on the left.

Next, the DNA sequence of the recombinant virus was compared with that of the parental virus, P-Oka. The recombinant viral DNA of rV02 was digested with *Bam*HI, separated by gel electrophoresis and the restriction enzyme pattern was compared with that of P-Oka (Fig. 3). The results comfirmed that the VZV-BAC lacks the 8.1-kbp *Bam*HI wildtype fragment but contains two additional *Bam*HI fragments of approximately 7.8 and 9.2 kbp (Fig. 3, lane 2). These fragments resulted from the insertion of the BAC vector sequences. In rV02, from which the BAC vector sequences had been removed, the 8.1 kbp wild type fragment of P-Oka migrated at a slightly higher position because of the remaining *lox*P sequence (Fig. 3, lane 3).

3.4. BAC-derived virus rV02 displays wild-type like in vitro growth properties

To compare the growth of the recombinant virus, rV02, with that of P-Oka, the infectious center assay was performed as described previously [2]. Briefly, HEL cells were infected with rV02 or P-Oka, then harvested by trypsin treatment 0-5 days after infection. The trypsin-treated cells were then diluted and transferred onto monolayers of HEL cells, and the numbers of infected cells were assessed by count-

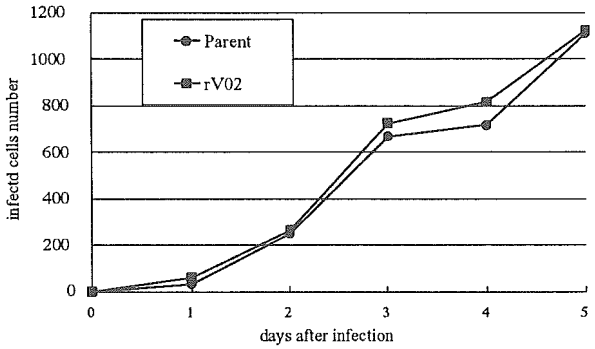

Fig. 4. Infectious center assay of P-Oka and rV02. HEL cells in 35-mm dishes were infected with rV02 or the wild-type strain, P-Oka at a multiplicity of 0.01 PFU per cell, then the cells were washed and treated with trypsin at 0–5 days PI. The trypsin-treated cells were diluted and transferred onto a monolayer of newly prepared HEL cells in 35-mm dishes, and the numbers of infected cells were assessed by counting the number of VZV plaques appearing at 7 days postinfection. The number of infected cells was normalized to the initial viral titer per dish; the fold increase indicates the number of infected cells to which virus had spread from one initial infected cell at day 0.

ing the plaques 7 days after inoculation. As shown in Fig. 4, rV02 displayed similar growth compare to P-Oka in this assay. These results suggested that the in vitro growth of the BAC-derived recombinant virus, rV02, was indistinguishable from that of P-Oka.

There are several reports describing the cloning of a herpesvirus genome as an infectious bacterial artificial chromosome in *E. coli* [3–20]. However, to date there has been no report on the cloning of the VZV genome as an infectious BAC in *E. coli*. The propagation of VZV virions in tissue culture is difficult because of the highly cell-associated nature of the virus and low cell-free virus production in the culture supernatant. In addition, it is difficult to isolate viral DNA containing the full-length genome. Therefore, the function of VZV proteins has been largely analyzed by analogy to herpes simplex virus type 1, the prototypical neurotropic human alphaherpesvirus [27]. Recombinant VZV genomes have been reconstituted using cosmid DNAs [28]. The cosmid system is useful for studying the role of the viral genome and leads directly to the generation of recombinant viruses. However, the subgenomic fragments have to be released from the vector backbone prior to transfection and several recombination events are required in eukaryotic cells for reconstitution.

Here, we have described construction of the first infectious BAC clone for VZV in *E. coli* and successful reconstitution of recombinant virus from the full-length VZV BAC genome for the first time. We have described construction of the first infectious clone for VZV in *E. coli*. This system will be a very useful tool for analyzing the function of VZV-encoded genes by mutagenesis of VZV BAC in *E. coli*, using established protocols [21],and is likely to be more efficient and reliable than reconstituting the genes from overlapping cosmid fragments [10]. Furthermore, this method will considerably speed up the construction of VZV mutants, and allow studies on the functional role of VZV genes in host–virus interactions.

Acknowledgements

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References

[1] Takahashi M, Otsuka T, Okuno Y, Asano Y, Yazaki T. Live vaccine used to prevent the spread of varicella in children in hospital. Lancet 1974;2(7892):1288–90.
[2] Gomi Y, Sunamachi H, Mori Y, Nagaike K, Takahashi M, Yamanishi K. Comparison of the complete DNA sequences of the Oka varicella vaccine and its parental virus. J Virol 2002;76(22):11447–59.
[3] Adler H, Messerle M, Wagner M, Koszinowski UH. Cloning and mutagenesis of the murine gammaherpesvirus 68 genome as an infectious bacterial artificial chromosome. J Virol 2000;74(15):6964–74.
[4] Borst EM, Hahn G, Koszinowski UH, Messerle M. Cloning of the human cytomegalovirus (HCMV) genome as an infectious bacterial artificial chromosome in *Escherichia coli*: a new approach for construction of HCMV mutants. J Virol 1999;73(10):8320–9.
[5] Delecluse HJ, Hilsendegen T, Pich D, Zeidler R, Hammerschmidt W. Propagation and recovery of intact, infectious Epstein-Barr virus from prokaryotic to human cells. Proc Natl Acad Sci USA 1998;95(14):8245–50.
[6] Delecluse HJ, Kost M, Feederle R, Wilson L, Hammerschmidt W. Spontaneous activation of the lytic cycle in cells infected with a recombinant Kaposi's sarcoma-associated virus. J Virol 2001; 75(6):2921–8.
[7] Horsburgh BC, Hubinette MM, Qiang D, MacDonald ML, Tufaro F. Allele replacement: an application that permits rapid manipulation of herpes simplex virus type 1 genomes. Gene Ther 1999;6(5):922–30.
[8] Marchini A, Liu H, Zhu H. Human cytomegalovirus with IE-2 (UL122) deleted fails to express early lytic genes. J Virol 2001; 75(4):1870–8.
[9] McGregor A, Schleiss MR. Molecular cloning of the guinea pig cytomegalovirus (GPCMV) genome as an infectious bacterial artificial chromosome (BAC) in *Escherichia coli*. Mol Genet Metab 2001;72(1):15–26.
[10] Messerle M, Crnkovic I, Hammerschmidt W, Ziegler H, Koszinowski UH. Cloning and mutagenesis of a herpesvirus genome as an infectious bacterial artificial chromosome. Proc Natl Acad Sci USA 1997;94(26):14759–63.
[11] Rudolph J, Osterrieder N. Equine herpesvirus type 1 devoid of gM and gp2 is severely impaired in virus egress but not direct cell-to-cell spread. Virology 2002;293(2):356–67.
[12] Saeki Y, Ichikawa T, Saeki A, et al. Herpes simplex virus type 1 DNA amplified as bacterial artificial chromosome in *Escherichia coli*: rescue of replication-competent virus progeny and packaging of amplicon vectors. Hum Gene Ther 1998;9(18):2787–94.

[13] Schumacher D, Tischer BK, Fuchs W, Osterrieder N. Reconstitution of Marek's disease virus serotype 1 (MDV-1) from DNA cloned as a bacterial artificial chromosome and characterization of a glycoprotein B-negative MDV-1 mutant. J Virol 2000;74(23):11088–98.
[14] Smith GA, Enquist LW. Construction and transposon mutagenesis in *Escherichia coli* of a full-length infectious clone of pseudorabies virus, an alphaherpesvirus. J Virol 1999;73(8):6405–14.
[15] Smith GA, Enquist LW. A self-recombining bacterial artificial chromosome and its application for analysis of herpesvirus pathogenesis. Proc Natl Acad Sci USA 2000;97(9):4873–8.
[16] Stavropoulos TA, Strathdee CA. An enhanced packaging system for helper-dependent herpes simplex virus vectors. J Virol 1998; 72(9):7137–43.
[17] Tanaka M, Kagawa H, Yamanashi Y, Sata T, Kawaguchi Y. Construction of an excisable bacterial artificial chromosome containing a full-length infectious clone of herpes simplex virus type 1: viruses reconstituted from the clone exhibit wild-type properties in vitro and in vivo. J Virol 2003;77(2):1382–91.
[18] Wagner M, Jonjic S, Koszinowski UH, Messerle M. Systematic excision of vector sequences from the BAC-cloned herpesvirus genome during virus reconstitution. J Virol 1999;73(8):7056–60.
[19] Yu D, Smith GA, Enquist LW, Shenk T. Construction of a self-excisable bacterial artificial chromosome containing the human cytomegalovirus genome and mutagenesis of the diploid TRL/IRL13 gene. J Virol 2002;76(5):2316–28.
[20] Zhou FC, Zhang YJ, Deng JH, et al. Efficient infection by a recombinant Kaposi's sarcoma-associated herpesvirus cloned in a bacterial artificial chromosome: application for genetic analysis. J Virol 2002;76(12):6185–96.
[21] Wagner M, Ruzsics Z, Koszinowski UH. Herpesvirus genetics has come of age. Trends Microbiol 2002;10(7):318–24.
[22] Hirt B. Selective extraction of polyoma DNA from infected mouse cell cultures. J Mol Biol 1967;26(2):365–9.
[23] Shizuya H, Birren B, Kim UJ, et al. Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in *Escherichia coli* using an F-factor-based vector. Proc Natl Acad Sci USA 1992; 89(18):8794–7.
[24] Greaves RF, Brown JM, Vieira J, Mocarski ES. Selectable insertion and deletion mutagenesis of the human cytomegalovirus genome using the *Escherichia coli* guanosine phosphoribosyl transferase (gpt) gene. J Gen Virol 1995;76(Pt 9):2151–60.
[25] Zhang Y, McKnight JL. Herpes simplex virus type 1 UL46 and UL47 deletion mutants lack VP11 and VP12 or VP13 and VP14, respectively, and exhibit altered viral thymidine kinase expression. J Virol 1993;67(3):1482–92.
[26] Kanegae Y, Lee G, Sato Y, et al. Efficient gene activation in mammalian cells by using recombinant adenovirus expressing site-specific Cre recombinase. Nucleic Acids Res 1995;23(19):3816–21.
[27] Cohrs RJ, Wischer J, Essman C, Gilden DH. Characterization of varicella-zoster virus gene 21 and 29 proteins in infected cells. J Virol 2002;76(14):7228–38.
[28] Cohen JI, Seidel KE. Generation of varicella-zoster virus (VZV) and viral mutants from cosmid DNAs: VZV thymidylate synthetase is not essential for replication in vitro. Proc Natl Acad Sci USA 1993;90(15):7376–80.

6. 麻疹，風疹，水痘の病態と予防

多屋馨子*

要　旨

麻疹，風疹，水痘はワクチンによる予防可能疾患であるが，国内の予防接種率は十分ではない。定期予防接種対象疾患である麻疹，風疹については，近年の感染症対策により患者数は減少しているものの，小児科定点からの報告のみで年間数千人である。2005年4月から，定期予防接種の改正が予定されている。一方，水痘は，任意接種対象疾患であり，小児科定点からの報告のみで年間約25万人である。3疾患ともに，通常，小児に好発するが，成人での発症も近年問題になっている。

Key words　予防接種，定期接種，任意接種，先天性風疹症候群

はじめに

一般に，麻疹，風疹，水痘は小児の疾患と考えられる傾向にある。しかし，これら3疾患は決して小児のみが罹患する疾患ではない。成人が麻疹に罹患し重症化あるいは死亡した報告[1]，妊娠中の風疹罹患による先天性風疹症候群の発生[2]，水痘の重症化による成人の入院[3]など，成人での発症が近年問題になっており，小児での流行を抑制することに加えて，年齢を小児に限定しない対策が必要である。

これら3疾患はいずれも，有効なワクチンが開発され，国内で市販・流通しているにもかかわらず，その接種率は決して満足できるものではない。小児の定期接種対象疾患である麻疹・風疹の予防接種率も未だ十分でなく，任意接種対象疾患である水痘の小児における予防接種率は，20%程度と極めて低い。

本稿では，3疾患の病態について概説するとともに，これら3疾患の予防法について取り上げてみたい。

I. 麻　　疹

麻疹の原因ウイルスである麻疹ウイルスは；*Paramyxoviridae Morbillivirus* に属し，エンベロープを有する一本鎖RNAウイルスである。リン

Condition and prevention of measles, rubella, and chicken pox.

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パ組織に特異的に発現する膜蛋白であるSLAM (signaling lymphocyte activation molecule ; CDw 150)をレセプターとして感染する[4]。

感受性者がウイルスの曝露を受けると90%以上が感染・発症する程度の高い感染力を有する。わが国では，通常春から夏にかけて流行するが，2001年の流行以降，全国的な麻疹ワクチンキャンペーンが功を奏し，2004年の全国約3,000の小児科定点からの患者報告数は2001年の約20分の1まで減少し，従来見られていた季節的なピークは認められなくなっている。報告患者年齢は1歳が最も多く，0～4歳が好発時期である(**図1**)。成人麻疹については，全国約500の基幹定点から患者数が報告されているが，小児の流行状況とほぼ一致している。報告患者年齢は**図2**に示すように，20代前半にピークがあり10代後半から30代に多い。

A. 症　　状

約10～12日の潜伏期を経て発症する。38℃前後の発熱が2～4日間続くとともに，咳嗽，鼻汁，結膜充血，眼脂などのカタル症状が徐々に増悪する(カタル期)。下痢を伴うことも多い。この時期が最も感染力が強い。発疹出現1～2日前に，頬粘膜に麻疹に特徴的な白色の粘膜疹(コプリック斑)が出現するが，発疹出現後は急速に消失する。発熱は一旦下がったかのように見えるが半日程度で再び39℃以上の高熱となり，耳後部，頸部，前額部より特徴的な発疹が出現し，顔面，体幹部，上下肢に広がる(発疹期)。カタル症状は益々増悪し，39℃以上の発熱は発疹出現後も3～4日間続く。特徴的な麻疹顔貌を呈する。発疹は融合して斑丘疹となるが，一部に健常皮膚を残す。その後，暗赤色となり，色素沈着を残して回復期に向かう。合併症を併発しなかった場合は，通常7～10日後に回復する。

合併症として頻度の高いものに肺炎があるが，脳炎と並んで麻疹の二大死因と言われている。麻疹ウイルスによる肺炎，細菌の二次感染による肺炎が中心であるが，細胞性免疫不全患者に見られる巨細胞性肺炎は，予後不良である。中耳炎は，細菌の二次感染により生じ，麻疹患者の約5～15%にみられる。喉頭炎および気管支炎も比較的よくみられる合併症である。

稀な合併症として，心筋炎，心外膜炎，脳炎がある。脳炎は，麻疹患者1,000例に0.5～1例の割合で合併し，発疹出現後2～6日頃に発症することが多い。患者の約20～40%に中枢神経系の後遺症を残し，死亡率は約15%である。遅発性の中枢神経合併症として，亜急性硬化性全脳炎(subacute sclerosing panencephalitis : SSPE)があるが，発症から平均6～9ヵ月で死亡する予後不良の疾患である。知能障害，性格の変化，運動障害などが徐々に進行し，錐体・錐体外路症状を呈した後，自発運動が不可能となる。0歳児での麻疹発症，免疫不全状態での発症者に多いと言われている。

B. 診 断 法

従来，特徴的な臨床症状から診断されることが多かったが，患者数が減少するとともに，麻疹ウイルス感染症としての実験室内診断が益々重要となる。

血液あるいは咽頭ぬぐい液からの麻疹ウイルスの分離・同定は，Kobuneらによる B95a細胞の確立により，早ければ24時間以内に可能となった[5]。また，magnetofectionを用いて，SLAM発現Vero細胞での麻疹ウイルス分離も最近Kadotaらによって報告された。RT-PCR法を用いて，血液あるいは咽頭ぬぐい液から麻疹ウイルスgenomeを検出する方法も用いられるが，いずれも健康保険の適用はなく，研究室レベルでの診断方法である。しかし，近年の流行ウイルス株を調べたり，ウイルスの変異などを検討するには，これらの方法は重要である。通常，カタル期から発疹出現後3日以内のウイルス分離率が高い。

健康保険適用がある方法としては，麻疹特異的IgM抗体価の測定，急性期と回復期のペア血清での麻疹IgG抗体の陽転あるいは有意上昇をもって診断可能である。抗体測定方法には，赤血球凝集抑制反応(hemagglutination inhibition : HI)法，補体結合反応(complement fixation : CF)

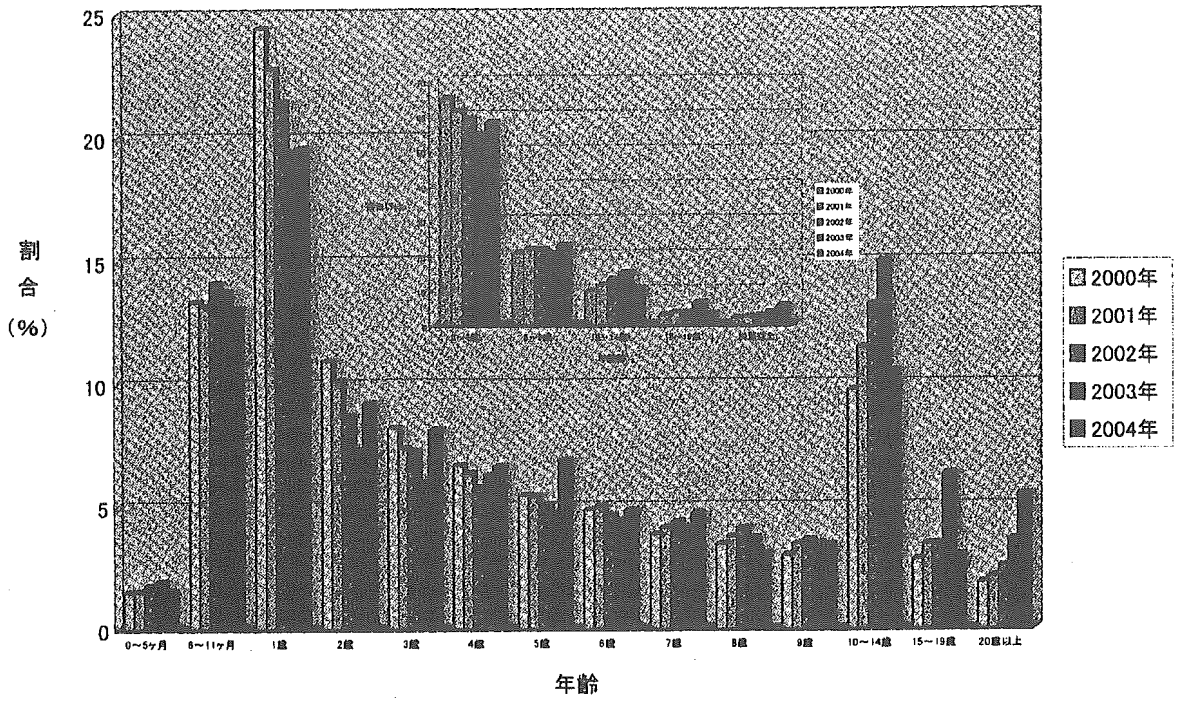

図1　麻疹報告患者年齢別割合（2000～2004 年）－感染症発生動向調査より－

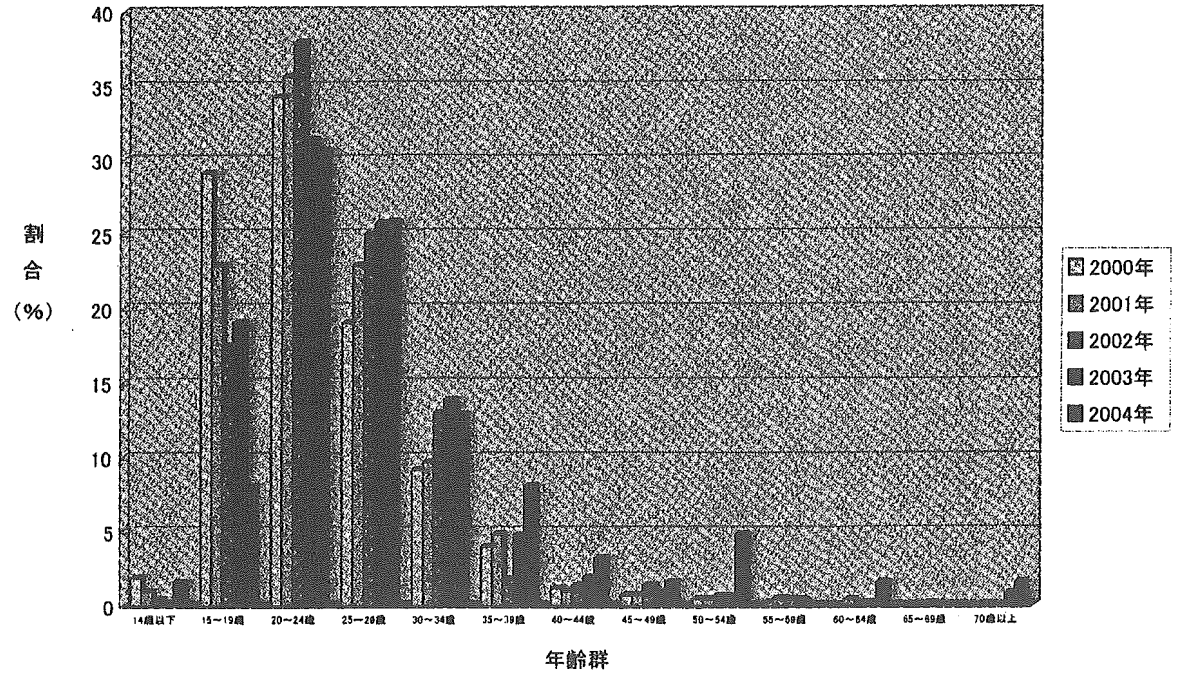

図2　成人麻疹報告患者年齢別割合（2000～2004 年）－感染症発生動向調査より－

法，中和法，ゼラチン粒子凝集反応（particle agglutination：PA）法，ELISA 法などが用いられているが，HI 法，CF 法の感度は，中和法，PA 法，ELISA 法に比して低い。

C. 治療および予防法

特異的な治療法はなく，ワクチンによる予防