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武田博明	高齢者でPPV23が定期接種化され、PCV13が適応拡大されました。23価があるのになぜ13価があるのでしょうか？	ワクチンジャーナル	3(1)	30-31	2015
西　順一郎	Hib・肺炎球菌ワクチンの普及は抗菌薬適正使用を促進する	日本小児臨床薬理学会雑誌	28(1)	43-46	2015
西　順一郎	Hibワクチン・肺炎球菌ワクチン　特集　小児感染症2015－小児感染症のマネジメント	小児科臨床	68(12)	2637-2642	2015
渡邊　浩	特集/急増する肺炎死亡とその対策、耐性菌の現状と対策	臨牀と研究	92(12)	1571-1574	2015
渡邊　浩	特集：成人の予防接種はどうあるべきか？－予防医療推進の観点から－、種々の状況下における予防接種のあり方、海外渡航時に必要なワクチン－短期滞在者－	診断と治療	103(11)	1475-1479	2015

Ⅳ. 研究成果の刊行物・別刷

7 海外渡航者に対する予防接種（成人）

summary

わが国の海外渡航者数は増加し，感染症に罹患する危険性が増しているが，海外渡航時のワクチンを接種できる医療機関は多くない。ワクチン接種の環境整備を行い，海外渡航者に対する感染症対策を啓発すべきである。

近年，わが国の海外渡航者数は増え続け，年間1,800万人以上になっている。渡航先や旅行形態にも変化がみられ，仕事のため家族連れで長期間途上国に赴任する場合や，既存の観光地のみならず冒険旅行などのように従来とは異なる地域に足を踏み入れる場合なども多くなっており，海外渡航者が様々な感染症に罹患する危険性が増している（表1）[1]。

欧米諸国では，海外渡航者の健康問題を扱う医療機関としてトラベルクリニックが数多く設置されており，健康指導，ワクチン接種や携帯医薬品の処方などが行われているが[2]，わが国においてトラベルクリニックは，都市部においては増えているものの地方ではまだ少なく，地域によっては海外渡航時のワクチンを接種できる医療機関がほとんどないという場合もめずらしくないのが現状である。本来，海外渡航をする人は渡航地の感染症情報や治安状況を事前に調べ，ワクチン接種をはじめとする必要な感染症予防対策を準備しておく必要があるが，残念ながら多くの日本人にはまだそのような習慣はない。

(1) 健康問題の発生頻度と感染症の種類

途上国に1カ月間滞在した場合，何らかの健康問題が半数以上の渡航者に発生するとされている。これには疲労や不眠など軽い症状も含むが，下痢や感冒といった実際の病気に罹る頻度は20～30%である。また発熱や下痢などの症状で渡航先あるいは帰国後医療施設を受診する頻度は8%，死亡する頻度は0.001%とされている[3]。このように死に至る頻度は決して高くない

表1　海外で罹りやすい感染症

感染経路	生活上の注意	感染症	主な流行地域	主な症状	ワクチンの有無
飲食物から感染	・ミネラルウォーターを飲む ・加熱した料理を食べる	旅行者下痢症	発展途上国	下痢，嘔吐	
		A型肝炎	発展途上国	発熱，黄疸，全身倦怠感	○
		ポリオ	南アジア，アフリカ	発熱，手足の麻痺	○
		腸チフス	発展途上国（特に南アジア）	発熱，腹痛	○*
		細菌性赤痢	発展途上国	発熱，腹痛，下痢，血便	
		コレラ	発展途上国	水様性下痢，脱水症状	○*
患者の飛沫などで感染	・手洗いやうがい ・人ごみを避ける	インフルエンザ	全世界	発熱，咽頭痛	○
		結核	発展途上国	咳・痰，体重減少	○
		髄膜炎菌性髄膜炎	西アフリカなど	発熱，意識障害，頭痛	○*
蚊が媒介	・皮膚を露出しない ・昆虫忌避剤を塗る ・殺虫剤を散布する	マラリア	発展途上国（熱帯・亜熱帯）	発熱，悪寒	
		デング熱	東南アジア，中南米	発熱，発疹	
		日本脳炎	アジア	発熱，意識障害	○
		黄熱	熱帯アフリカ，南米	発熱，黄疸	○
性行為で感染	・行きずりの性行為を控える ・医療行為にも注意	B型肝炎	アジア，アフリカ，南米	発熱，黄疸，全身倦怠感	○
		梅毒	発展途上国	性器潰瘍，皮疹	
		HIV感染症	全世界（特に発展途上国）	発熱，リンパ節腫脹	
動物から感染	・動物に近寄らない	狂犬病	全世界（特に発展途上国）	恐水発作，けいれん	○
傷口から感染	・傷口を消毒する	破傷風	全世界	口が開かない，けいれん	○

*：腸チフス，コレラ，髄膜炎菌性髄膜炎にはワクチンがあるが，日本では未承認である

（文献1より一部改変）

ものの，何らかの健康問題が発生したり，病気になったりする頻度は比較的高い。図1に途上国への旅行者における各感染症の1カ月間の推定罹患率を示す[4]。途上国への渡航により罹患する感染症としては，旅行者下痢症やA型肝炎などの水や食物に関連した感染症が最も多く，それについでマラリアやデング熱などの蚊が媒介する疾患，感冒やインフルエンザなどのヒトからヒトに伝播する呼吸器感染症，B型肝炎やHIV感染などの性行為感染症などが挙げられる。

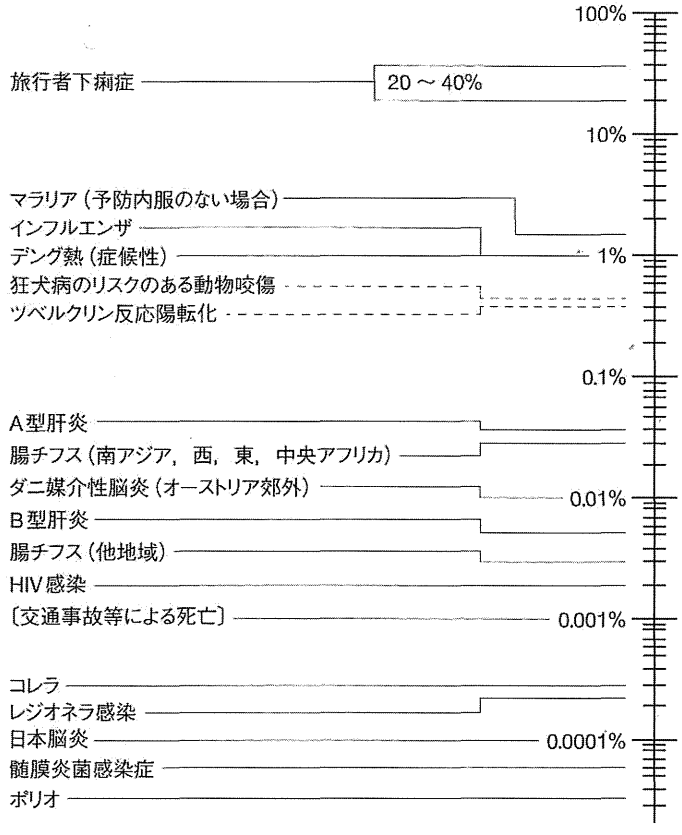

図1　途上国への旅行者における各感染症の1カ月間の推定罹患率（2010年）
（文献4より改変）

(2) 海外への渡航を控えた成人に必要なワクチン

外国に滞在中，特に途上国への渡航者はその地域にみられる各種の感染症の危険に曝されるため，できる限り適切なワクチンを受けておくことが望ましい。ワクチンを選ぶ際には，海外渡航に際してのワクチン接種に関する情報源としてよく用いられる厚生労働省検疫所のホームページ『FORTH―海外で健康に過ごすために』(http://www.forth.go.jp/) や，市販されている地域別の感染症情報のソフト (Tropimed®，Travax® など) を参考に，単に目的地だけでなく，渡航期間，渡航の形態，宿泊施設，職種など様々な因子を考慮する必要がある。もちろん経済的な事情にも配慮する。

海外渡航時のワクチンは，①麻疹やポリオなど自らの感染予防のみならず周囲への感染を防止するため主に小児期より定期接種するもの，②黄熱ワクチンのように国際保健規則 (International Health Regulations：IHR) に基づき入国時に予防接種証明書を要求されることがあるもの，③A型肝炎，B型肝炎，破傷風，狂犬病，日本脳炎など渡航先で流行している感染症で，わが国では存在しないか，感染する危険性が少ない病気を予防するという個人防衛の意味があるものの3種類がある。

接種部位を別にして同時に複数のワクチンを接種する「同時接種」は認められているが，ワクチンには，弱めた病原体そのものを用いる生ワクチンと，死滅・不活化した病原体や毒素を用いる不活化ワクチンやトキソイドがあり，同時接種でない場合には，次のワクチン接種までには生ワクチンで27日以上，不活化ワクチンやトキソイドで6日以上の間隔をあける必要があるので注意を要する[5]。またワクチンによって接種の回数，効果の持続期間が異なるので，常にワクチンの記録は怠らず，長期間の免疫を保つために次回のワクチン接種の時期を知っておくことも大切である。

(3) わが国における海外渡航時のワクチンの問題点

現在，海外で通常に使用されているワクチンの多くが国内では未承認である。腸チフス，髄膜炎菌，コレラ，ダニ媒介性脳炎ワクチンなどは海外で接種するか，あるいは国内では輸入代行業者などを通じ個人輸入している医療

機関でしか接種できないのが現状である。また，狂犬病ワクチンやA型肝炎ワクチンなどの国産ワクチンの品薄が慢性的に持続している，ワクチンを接種できる医療機関が十分に整備されていない，海外渡航者に対するワクチンの必要性の啓発が十分にできておらず渡航者が海外に比べ積極的にワクチン接種を行わないなどの問題があり，海外渡航者のためのワクチン接種の環境は十分に整っているとは言えない状況である。

日本渡航医学会は，2010年，海外渡航者にとって本来必要なワクチンを大きな支障なく接種できるようにすることを目的として『海外渡航者のためのワクチンガイドライン2010』を発刊した[6]。本ガイドラインには各ワクチンの解説だけでなく，接種法についてのわが国と国際基準の比較，法律的事項，ワクチン基礎講座も示されている。

都道府県別の予防接種実施機関は前述のFORTHで検索することができる。また日本渡航医学会のホームページ(http://www.tramedjsth.jp/)では国内のトラベルクリニックのリストが公開されており，診療時間，未承認ワクチンを含め取り扱っているワクチンの種類，海外健診，英文診断書作成，高山病・マラリアの予防内服処方，帰国後診療の可否やクリニックの特徴などについて詳細に掲載されている。また，日本渡航医学会は2011年より，「トラベルクリニックサポート事業」を開始し，トラベルクリニックは地方においても徐々に増えてきている。

(4) まとめ

日本人が以前より気軽に海外渡航するようになり，渡航地に存在する感染症に罹患する機会は今後も増加することが予想される。楽しい旅をするには渡航前に観光，ショッピングなどの情報収集だけではなく，健康や安全への備えが大切である。ワクチンですべての病気を防ぐことはできないが，少なくとも渡航地に存在し，罹患率の高い疾患，重症化しやすい疾患あるいは致命率の高い疾患でワクチンにより予防可能な疾患については，事前のワクチン接種を検討すべきと思われる。今後わが国における海外渡航者のためのワクチンの環境整備が向上するとともに，海外渡航者が事前に渡航地の感染症情報を収集し，必要な感染症対策を準備する習慣を持てるよう啓発していくべきであろう。

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渡邊 浩

MAJOR ARTICLE

A Bivalent Vaccine Based on a PB2-Knockout Influenza Virus Protects Mice From Secondary Pneumococcal Pneumonia

Ryuta Uraki,[1,a] Zhenyu Piao,[4,a] Yukihiro Akeda,[4] Kiyoko Iwatsuki-Horimoto,[1] Maki Kiso,[1] Makoto Ozawa,[5,6] Kazunori Oishi,[3,4] and Yoshihiro Kawaoka[1,2,7,8]

[1]Division of Virology, Department of Microbiology and Immunology, [2]Department of Special Pathogens, International Research Center for Infectious Diseases, Institute of Medical Science, University of Tokyo, [3]Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo, [4]Laboratory of Clinical Research on Infectious Diseases, International Research Center for Infectious Diseases, Research Institute for Microbial Diseases, Osaka University, [5]Laboratory of Animal Hygiene, [6]Transboundary Animal Diseases Center, Joint Faculty of Veterinary Medicine, Kagoshima University, [7]ERATO Infection-Induced Host Responses Project (JST), Saitama, Japan; and [8]Department of Pathobiological Sciences, University of Wisconsin-Madison

Background. Secondary bacterial infections after influenza can be a serious problem, especially in young children and the elderly, yet the efficacy of current vaccines is limited. Earlier work demonstrated that a replication-incompetent PB2-knockout (PB2-KO) influenza virus possessing a foreign gene in the coding region of its PB2 segment can serve as a platform for a bivalent vaccine.

Methods. In the current study, we generated the PB2-KO virus expressing pneumococcal surface protein A (PspA), PB2-KO-PspA virus, the replication of which is restricted to PB2-expressing cells. We then examined the protective efficacy of intranasal immunization with this virus as a bivalent vaccine in a mouse model.

Results. High levels of influenza virus–specific and PspA-specific antibodies were induced in the serum and airways of immunized mice. The intranasally immunized mice were protected from lethal doses of influenza virus or *Streptococcus pneumoniae*. These mice were also completely protected from secondary pneumococcal pneumonia after influenza virus infection.

Conclusions. These findings indicate that our recombinant influenza virus serves as a novel and powerful bivalent vaccine against primary and secondary pneumococcal pneumonia as well as influenza.

Keywords. secondary pneumococcal pneumonia; replication-incompetent influenza virus; bivalent vaccine.

Secondary bacterial infections after influenza account for a sizeable proportion of the deaths associated with influenza pandemics [1, 2]. Most secondary bacterial infections are caused by *Streptococcus pneumoniae*.

S. pneumoniae is one of the major causes of disease and death resulting from pneumonia, bacteremia, and meningitis worldwide [3]. Pneumococcal carriage is considered to be an important source of the horizontal spread of this pathogen, because preceding nasopharyngeal colonization with this bacterium is essential for pneumococcal diseases [4]. *S. pneumoniae* is classified into >90 serotypes defined based on the antigenicity of their capsular polysaccharide, which is a virulence factor of this pathogen [5].

The current first line of defense against pneumococcal pneumonia is vaccination: pneumococcal polysaccharide conjugate vaccines derived from multiple serotypes of *S. pneumoniae* are used globally to provide protective immunity in infants [6, 7]. Although the introduction of the 7-valent pneumococcal conjugate vaccine resulted in significant declines in the incidence of invasive pneumococcal disease (IPD) caused by the serotypes covered by the vaccine, serotype replacement in carriage and IPD occurred [8–13]. Moreover, since the

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[a]R. U. and Z. P. contributed equally to this work.

Correspondence: Yoshihiro Kawaoka, DVM, PhD, Division of Virology, Department of Microbiology and Immunology, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku, Tokyo, Japan (kawaoka@ims.u-tokyo.ac.jp).

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introduction of a 13-valent pneumococcal conjugate vaccine for use in children, the frequency of serotypes not included in a 13-valent pneumococcal conjugate vaccine has increased in pediatric and adult patients with IPD [14, 15]. Therefore, an alternative vaccine format is desired for the control of *S. pneumoniae* infection.

Recent studies on pneumococcal vaccine development have focused on pneumococcal surface protein A (PspA), a choline-binding protein exposed on the cell surface of all pneumococcal strains [16–20]. Anti-PspA antibodies are known to overcome the anticomplementary effect of PspA, allowing for increased complement activation and C3 deposition on PspA-bearing bacteria [21–23]. In addition, anti-PspA antibodies enhance bacterial clearance and induce cross-serotype immunity [24–27]. Collectively these data suggest that PspA is a promising vaccine candidate against pneumococcal infection.

To prevent influenza, both inactivated and live attenuated vaccines are available [28, 29]. Inactivated vaccines present few safety concerns and are used globally; however, they do not induce the mucosal immune responses that play important roles in preventing influenza virus replication [30, 31]. Live attenuated vaccines elicit mucosal immune responses more efficiently than inactivated vaccine; however, their usage is restricted because of safety concerns [32–34]. To overcome the limitations of the current influenza vaccines, Ozawa et al [35] previously generated a replication-incompetent influenza virus that does not express the PB2 protein, an influenza virus polymerase subunit that is essential for virus replication. Mice intranasally immunized with PB2-knockout (PB2-KO) virus efficiently elicited mucosal immunity and were protected from challenge with a lethal dose of influenza virus [36, 37]. Uraki et al [37] also demonstrated the protective efficacy as bivalent vaccines of PB2-KO viruses by introducing foreign genes into their PB2-coding region. Together, these findings suggest that PB2-KO influenza virus is a novel platform for a bivalent influenza vaccine that is safe and efficacious.

In the current study, we generated PB2-KO virus expressing PspA as a bivalent vaccine for influenza and pneumococcal pneumonia and examined whether intranasal immunization with this bivalent vaccine could induce influenza virus–specific and PspA-specific antibodies and afford protection from lethal infection with influenza virus or *S. pneumoniae* in a mouse model.

METHODS

Cells

Human embryonic kidney cell (293T cell) were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum (Gibco). Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) supplemented with 5% newborn calf serum (NCS) (Equitech-Bio). AX4 cells, an MDCK-derived cell line with enhanced expression of human-type receptors for influenza virus [38], were maintained in 5% NCS-MEM supplemented with puromycin (2 μg/mL). AX4/PB2 cells, which are AX4 cells stably expressing the PB2 protein derived from A/Puerto Rico/8/34 (H1N1; PR8) [35], were maintained in 5% NCS-MEM supplemented with puromycin (2 μg/mL) and blasticidin (10 μg/mL). All cells were maintained in a humidified incubator at 37°C with 5% carbon dioxide.

Viral and Bacterial Strains

H1N1 subtype influenza virus PR8 strain was propagated in MDCK at 37°C for 48 hours and harvested as culture supernatants. A/New Caledonia/20/99 (H1N1; NC) virus was obtained from the Research Foundation for Microbial Diseases, Osaka University. *S. pneumoniae* WU2 strain (serotype 3) [39], which expresses PspA (family 1, clade 2) and is virulent in mice, and EF3030 strain (serotype 19F) [40], which expresses PspA (family 1, clade 1) and is relatively avirulent in mice, were grown in Todd-Hewitt broth (BD) supplemented with 0.5% yeast extract (THY) at 37°C with 5% carbon dioxide. The stocks of the bacterial strains for the challenge experiments were collected at an optical density (OD) at a wavelength of 600 nm (OD_{600}) of 0.3–0.4, washed with fresh THY, resuspended in fresh THY with 10% glycerol, and stored at −80°C until use.

Plasmid-Driven Reverse Genetics

The wild-type PR8 and PB2-KO viruses were engineered by using reverse genetics, as described elsewhere [41]. For the expression of viral RNA, plasmids containing the cloned complementary DNAs of PR8 genes between the human RNA polymerase I promoter and the mouse RNA polymerase I terminator (referred to as PolI plasmids) were used. To generate the PR8-based PB2-KO virus possessing the antigenic portion of PspA in the recombinant PB2 gene (PR8/PB2-PspA virus) in place of the authentic PB2 gene, pPolIPB2(120)PspA(336) (Figure 1*A*) and the remaining 7 PolI plasmids were cotransfected into 293T cells along with eukaryotic protein expression plasmids for PB2, PB1, PA, and NP proteins derived from PR8 virus by use of the TransIT 293 transfection reagent (Mirus), according to the manufacturer's instructions as described elsewhere [37]. The inserted antigenic portion of PspA was 302 amino acids (positions 32–333) of mature PspA belonging to family 1, clade 2 [42]. At 48 hours after transfection, the supernatants containing the PB2-KO virus were harvested and propagated in AX4/PB2 cells. Viruses were titrated by means of plaque assays with AX4/PB2 cells.

Growth Kinetics and Virus Titration

To determine virus growth kinetics, triplicate wells of confluent AX4 or AX4/PB2 cells were infected with viruses at a multiplicity of infection of 0.001 at 33°C. After 1 hour of virus adsorption, cells were washed in MEM containing 0.3 % bovine serum albumin and overlaid with MEM containing L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (1.0 μg/mL). Supernatants

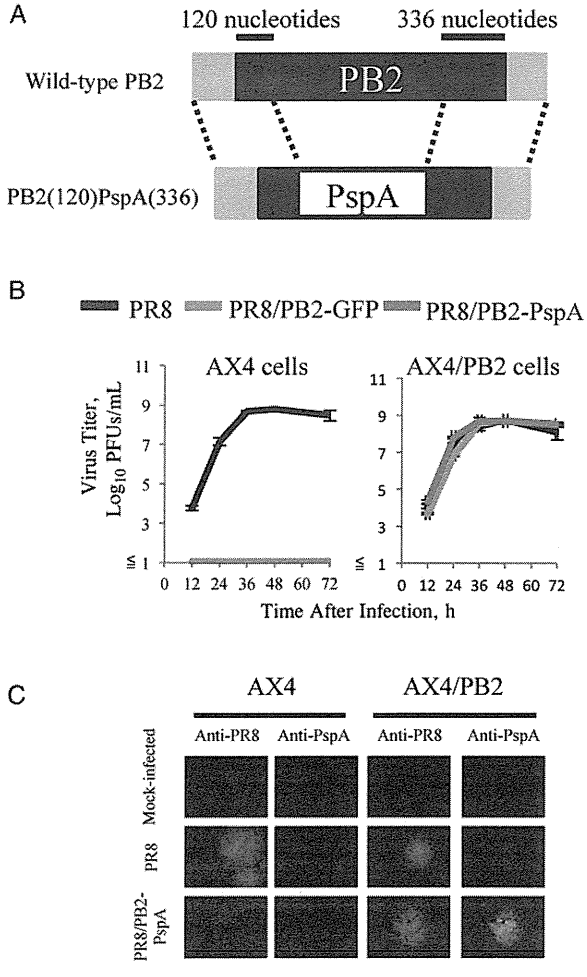

Figure 1. Characterization of PR8/PB2–pneumococcal surface protein A (PspA) virus. *A*, Schematic diagrams of wild-type PB2 and PB2(120)PspA (336) viral RNAs (vRNAs). PB2(120)PspA(336) vRNA possesses the 3′ noncoding region, 120 nucleotides of the coding sequence of PB2 vRNA, the PspA gene, and 336 nucleotides of the 3′ and 5′ noncoding regions of PB2 vRNA. The noncoding region and coding regions of PB2 vRNA are represented by gray and black bars, respectively. *B*, Growth kinetics of PR8/PB2–PspA virus. AX4 and AX4/PB2 cells were infected with PR8, PR8/PB2–green fluorescent protein (GFP), or PR8/PB2–PspA virus at a multiplicity of infection of 0.001. Supernatants were collected at 12, 24, 36, 48, and 72 hours after infection for virus titration by plaque assays in AX4/PB2 cells. *C*, PspA expression in AX4/PB2 cells infected with PR8/PB2–PspA virus. AX4 and AX4/PB2 cells were mock-infected or infected with PR8 or PR8/PB2–PspA virus. At 48 hours after infection, the cells were fixed and stained with anti-influenza polyclonal (R309) or anti-PspA polyclonal antibodies. Abbreviation: PFUs, plaque-forming units.

were collected at 12, 24, 36, 48, and 72 hours after infection and assayed for virus titers by means of plaque assays in AX4/PB2 cells.

Immunofluorescence Assay

Twenty-four hours after virus infection, cells were washed twice with phosphate-buffered saline and fixed with 4% paraformaldehyde for 15 minutes at room temperature. These cells were then stained with anti-influenza virus rabbit polyclonal (R309) and anti-PspA mouse polyclonal antibodies prepared in our laboratory.

Effect of Primary PR8/PB2-PspA Virus Inoculation of Mice on Secondary Infection

Five-week-old female C57BL/6 mice (Japan SLC) were anesthetized with isoflurane and inoculated with 50 μL of medium (MEM containing 0.3% bovine serum albumin fraction V), PR8/PB2–green fluorescent protein (GFP) virus (1.0×10^6 plaque-forming units [PFUs]) [35–37], PR8/PB2-PspA virus (1.0×10^6 PFUs), or NC virus (1.0×10^3 PFUs). Five days after virus inoculation, mice were intranasally challenged with 6.0×10^3 colony-forming units (CFUs) of *S. pneumoniae* WU2 strain in 30 μL of phosphate-buffered saline. Five days after infection with the WU2 strain, 6 mice were euthanized for quantitative bacterial cultures of lung tissue on sheep blood agar (BD). The survival of the remaining challenged mice (10 mice per group) was monitored daily for 10 days.

Immunization of Mice

Mice were intranasally inoculated with medium, PR8/PB2-GFP virus (1.0×10^6 PFUs), or PR8/PB2-PspA virus (1.0×10^6 PFUs) 3 times at 2-week intervals.

Detection of Virus-Specific Antibodies

Virus-specific antibodies in nasal wash, bronchoalveolar lavage fluid (BALF), and serum samples were detected using an enzyme-linked immunosorbent assay, as described elsewhere [36, 43]. The end-point titers were expressed as the reciprocal $\log_2$ of the last dilution, giving an OD_{405} of 0.1 OD unit above the OD_{405} of the negative control samples. Hemagglutination inhibition titers were determined as described elsewhere [44, 45].

Detection of PspA-Specific Antibodies

PspA-specific antibodies in serum, BALF, and nasal wash samples were detected using an enzyme-linked immunosorbent assay, as described elsewhere [46]. The end-point titers were expressed as the reciprocal $\log_2$ of the last dilution, giving an OD_{405} of 0.1 OD unit above the OD_{405} of the negative control samples.

Protection Tests

Lethal Influenza Virus Challenge

Two weeks after the final immunization, the immunized mice were intranasally challenged with 1.0×10^4 PFUs (10-fold 50% mouse lethal dose [10 MLD_{50}]) of PR8 virus (50 μL). Three and 6 days after the viral challenge, we euthanized 3 mice per group, collected the nasal turbinates and lungs, and determined the viral titers using plaque assays in AX4/PB2 cells. The survival of the remaining challenged mice (8 mice per group) was monitored daily for 14 days.

Lethal Pneumococcal Challenge

Two weeks after the final immunization, the immunized mice were intranasally challenged with 2.0×10^7 CFUs (3 MLD_{50}) of

S. pneumoniae WU2 strain (30 μL). The survival of the challenged mice (10 mice per group) was monitored daily for 10 days.

Secondary Pneumococcal Pneumonia

Two weeks after the final immunization, the immunized mice were intranasally infected with 1.0×10^3 PFUs of NC virus (30 μL). Three days after the viral challenge, we euthanized 6 mice per group, collected the nasal turbinates and lungs, and determined the viral titers using plaque assays in AX4/PB2 cells. Five days after the viral infection, the remaining mice were intranasally infected with 2.0×10^7 CFUs (3 MLD_{50}) or 6.0×10^3 CFUs (sublethal dose) of *S. pneumoniae* WU2 strain (30 μL). Five days after this bacterial challenge, we euthanized 6 mice per group, collected their lungs, and determined the bacterial densities on sheep blood agar, as described elsewhere [32, 47]. The survival of the remaining challenged mice (10 mice per group) was monitored daily for 10 days after the bacterial challenge.

Nasal Colonization

Two weeks after the final immunization, the immunized mice were intranasally challenged with 1.0×10^2 CFUs (sublethal dose) of *S. pneumoniae* EF3030 strain (30 μL). Five days after the bacterial challenge, we euthanized 10 mice per group, collected nasal wash samples, and determined the bacterial densities on sheep blood agar, as described elsewhere [32, 47].

Ethics Statement

Our research protocol for the use of mice followed the University of Tokyo's Regulations for Animal Care and Use, which was approved by the Animal Experiment Committee of the Institute of Medical Science, the University of Tokyo (approval No. PA10–15) and the Animal Care and Use Committee of the Research Institute for Microbial Diseases, Osaka University (approval No. Biken-AP-H23-05-0).

Statistical Analysis

Statistically significant differences in the bacterial densities or viral titers were assessed using 1-way analysis of variance followed by a Dunnett test. Log-rank statistical analysis was performed to determine significant differences in the survival rates of the immunized mice.

RESULTS

Characterization of the PR8/PB2-PspA Virus in Vitro

PR8-based PB2-KO virus expressing PspA (PR8/PB2-PspA virus) was generated by using plasmid-driven reverse genetics (Figure 1*A*). We first determined whether the PR8/PB2-PspA virus replicates only in PB2 protein-expressing and not normal cells. Although no replication of the PR8/PB2-PspA virus was detected in wild-type AX4 cells, as was also the case with the control PR8/PB2-GFP virus (Figure 1*B*, left), the replication of the PR8/PB2-PspA virus in AX4/PB2 cells was comparable to that of wild-type PR8 virus (Figure 1*B*, right). These results indicate that PR8/PB2-PspA virus replication was restricted to PB2 protein-expressing cells, as demonstrated elsewhere [35–37].

We then attempted to confirm that the PR8/PB2-PspA virus expressed PspA encoded in its PB2 gene in virus-infected

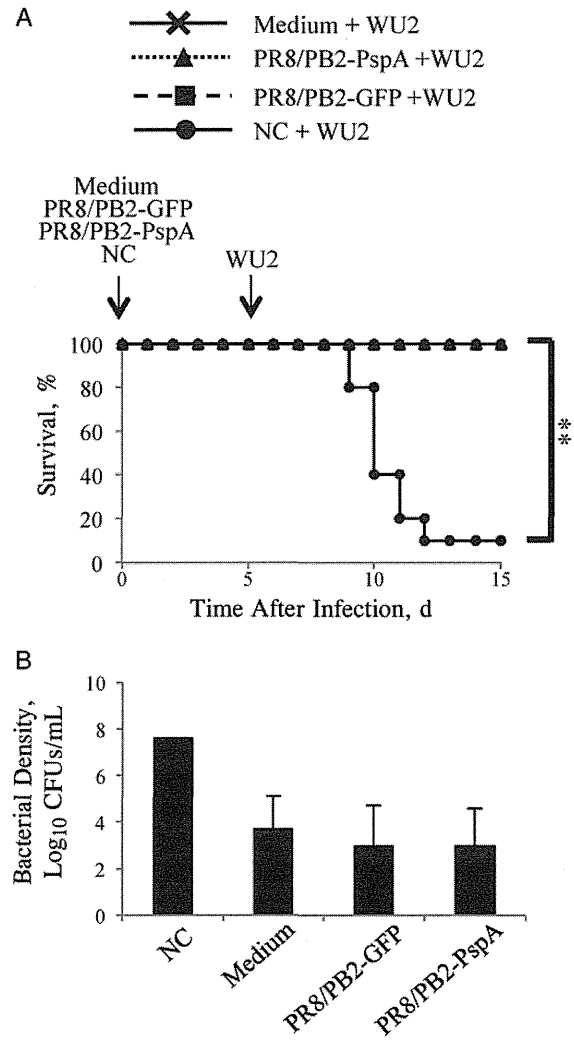

Figure 2. Survival of mice inoculated with PR8/PB2–pneumococcal surface protein A (PspA) virus and then infected with WU2 strain. Mice (n = 16) were mock-inoculated with medium, PR8/PB2–green fluorescent protein (GFP) (1.0×10^6 plaque-forming units [PFUs]), PR8/PB2-PspA (1.0×10^6 PFUs) or A/New Caledonia/20/99 (NC) (1.0×10^3 PFUs) virus. Five days after inoculation, the mice were intranasally challenged with WU2 strain (6.0×10^3 colony-forming units [CFUs]). *A*, Survival was monitored in 10 mice for 10 days after bacterial challenge. *B*, Lung tissues were collected 5 days after the bacterial challenge and subjected to bacterial titration on sheep blood agar. Values represent the mean (standard deviation) $\log_{10}$ CFUs per lung for 6 mice per group. Because 5 of the 6 mice inoculated with NC virus died after the challenge with WU2 strain, the lung bacterial density of the sole surviving mouse is shown.

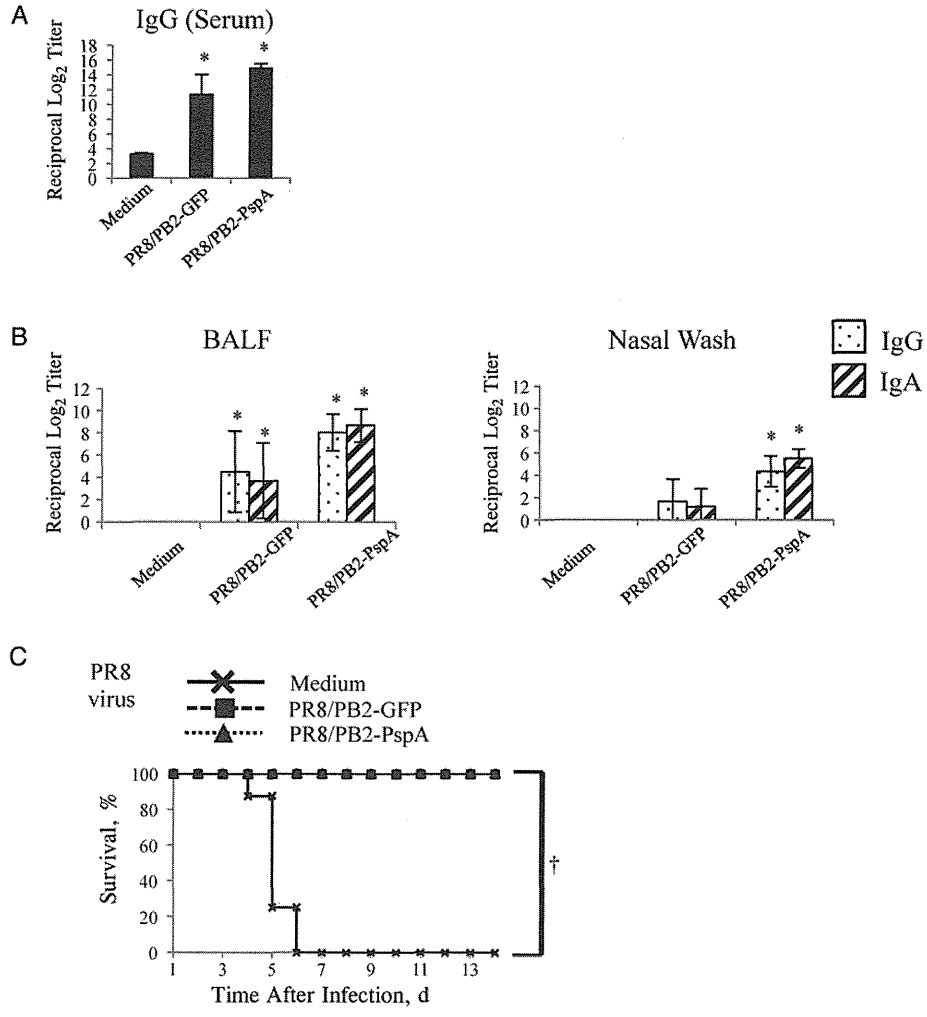

Figure 3. Antibody responses and survival of mice after influenza virus challenge. Mice were mock-immunized with medium or immunized with the PR8/PB2–green fluorescent protein (GFP) or PR8/PB2–pneumococcal surface protein A (PspA) virus 3 times at 2-week intervals. *A, B,* PR8-specific antibodies raised by the immunized mice were detected by means of an enzyme-linked immunosorbent assay with purified PR8 virus as an antigen. Immunoglobulin (Ig) G titers in the serum (*A*), IgG and IgA titers in bronchoalveolar lavage fluid (BALF) (*B,* left panel), and nasal wash samples (*B,* right panel) from mice intranasally mock-immunized with medium or immunized with the PR8/PB2-GFP or PR8/PB2-PspA virus were measured. Values are expressed as the mean (standard deviation [SD]) reciprocal $\log_2$ titer for 6 samples. $^*P < .05$. *C,* Two weeks after the final vaccination, mice were intranasally challenged with 10-fold 50% mouse lethal dose of PR8 virus. Survival was monitored in 8 mice for 14 days after challenge. $^\dagger P < .01$.

cells. Using an immunofluorescence assay, we detected both PspA and influenza viral proteins in AX4/PB2 cells, but not wild-type AX4 cells, infected with PR8/PB2-PspA virus (Figure 1*C*). Wild-type PR8 virus expressed viral proteins but-not PspA in both wild-type AX4 and AX4/PB2 cells. These results demonstrate that the PspA in the PB2 gene of the PR8/PB2-PspA virus was expressed in the virus-infected PB2-expressing cells.

Effect of Intranasal Inoculation of PB2-KO Virus on Susceptibility to *S. pneumoniae*

Previously, Ezoe et al [46] demonstrated that preinfection of mice with A/New Caledonia/20/99 (H1N1) (NC virus) increased their susceptibility to *S. pneumoniae* by about 3000-fold. We therefore examined whether preinoculation with either PR8/PB2-GFP virus or PR8/PB2-PspA virus increased the susceptibility of mice to the *S. pneumonia* WU2 strain. The survival rates of mice infected with PR8/PB2-GFP virus or PR8/PB2-PspA virus and then infected with a sublethal dose of *S. pneumoniae* WU2 strain (Figure 2*A*; 100% each) were significantly higher than that of mice preinfected with NC virus mice (10%). On day 5 after pneumococcal infection, the bacterial densities in the lungs of mice preinoculated with PR8/PB2-PspA virus were remarkably lower than those of the NC virus–preinfected mice and comparable to those in mock-inoculated and PR8/PB2-GFP virus–infected mice (Figure 2*B*). These results indicate

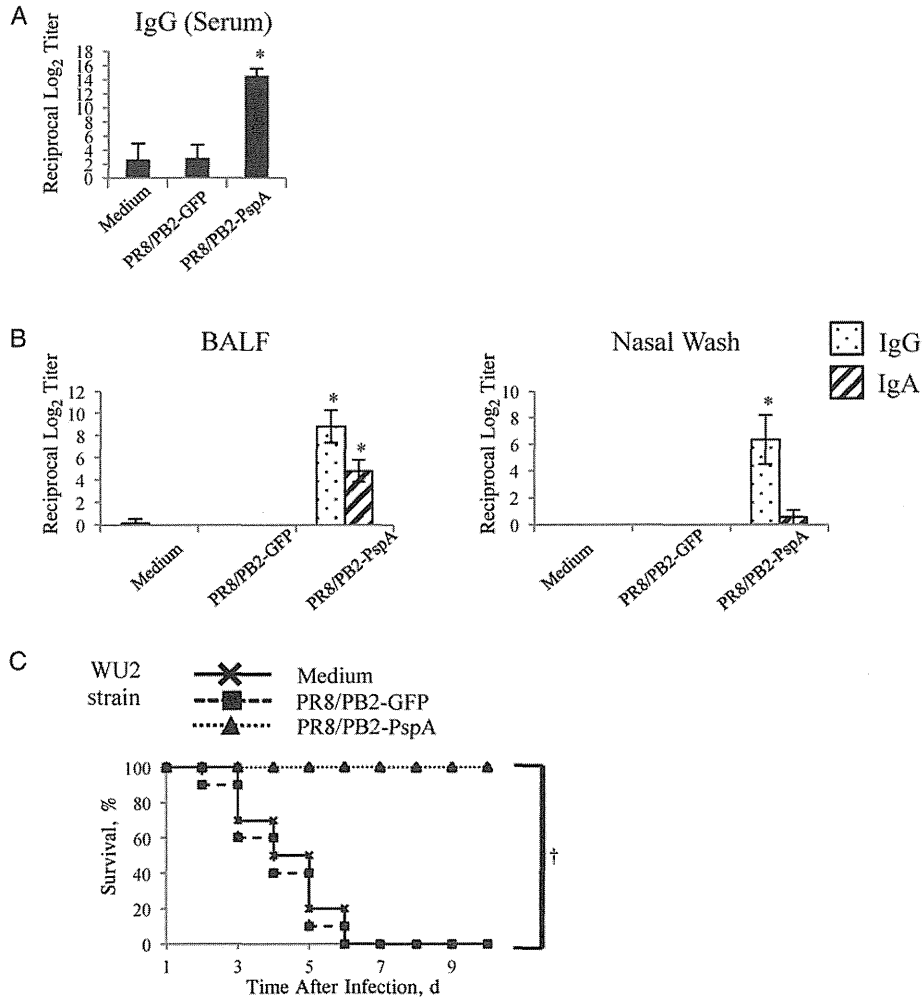

Figure 4. Antibody responses and survival of mice after *Streptococcus pneumoniae* challenge. Mice were mock-immunized with medium or immunized with the PR8/PB2–green fluorescent protein (GFP) or PR8/PB2–pneumococcal surface protein A (PspA) virus 3 times at 2-week intervals. *A. B*, PspA-specific antibodies raised by the immunized mice were detected by means of an enzyme-linked immunosorbent assay with PspA as an antigen. Immunoglobulin (Ig) G titers in serum (*A*), IgG and IgA titers in bronchoalveolar lavage fluid (BALF) (*B*, left panel), and nasal wash samples (*B*, right panel) from mice intranasally mock-immunized with medium or immunized with the PR8/PB2-GFP or PR8/PB2-PspA virus were measured. Values are expressed as the mean (standard deviation [SD]) reciprocal $\log_2$ titer of 6 samples. $^*P < .05$. *C*, Two weeks after the final vaccination, mice were intranasally challenged with 3-fold 50% mouse lethal dose of WU2 strain. Survival was monitored in 10 mice for 10 days after challenge. $^\dagger P < .01$.

that the PB2-KO virus inoculation did not alter the susceptibility of mice to *S. pneumoniae*.

Antibody Responses Induced by Intranasal Immunization With the PR8/PB2-PspA Virus

We examined the antibody responses in PR8/PB2-PspA virus–inoculated mice to PR8 virus, a backbone strain of PR8/PB2-PspA virus. As expected, both immunoglobulin (Ig) G and IgA against PR8 virus were efficiently elicited in the serum, BALF, and nasal wash samples of mice immunized with either PR8/PB2-GFP or PR8/PB2-PspA virus (Figure 3*A* and 3*B*).

We also determined whether PspA-specific antibodies were induced in mice immunized with the PR8/PB2-PspA virus. Although a negligible amount of PspA-specific IgG or IgA was detected in the serum, BALF, and nasal wash samples from the control mice, significantly higher levels of PspA-specific IgG or IgA were found in the serum, BALF, or nasal wash samples from mice immunized with the PR8/PB2-PspA virus (Figure 4*A* and 4*B*). These results indicate that PR8/PB2-PspA virus efficiently induced antibodies specific for influenza virus and PspA in the immunized mice.

Effect of Intranasal Immunization With the PR8/PB2-PspA Virus on Bacterial Clearance

We examined the bacterial colonization in the nasopharynx of the immunized mice by infecting them with an avirulent

S. pneumoniae EF3030 strain. On day 5 after the intranasal challenge, the bacterial densities in the nasal wash samples from mice immunized with the PR8/PB2-PspA virus (mean [standard deviation], 2.4 [0.9] $\log_{10}$ CFUs/mL) were significantly lower than those of mock-immunized or PR8/PB2-GFP virus–immunized mice (3.6 [0.7] and 3.6 [0.8] $\log_{10}$ CFUs/mL, respectively). These results indicate that the PR8/PB2-PspA virus immunization enhanced the clearance of *S. pneumoniae* from the nasopharynx.

Effect of Intranasal Immunization With the PR8/PB2-PspA Virus on a Lethal Infection With Influenza Virus and *S. pneumoniae*

We examined the protective efficacy of intranasal immunization with the PR8/PB2-PspA virus in a commonly used mouse influenza virus challenge model [48]. All mice immunized with either PR8/PB2-GFP or PR8/PB2-PspA virus survived (Figure 3*C*) after the challenge with 10 MLD_{50} of PR8 virus, whereas all mice mock-immunized with medium succumbed to the lethal infection with wild-type PR8 virus. In addition, mice immunized with either PR8/PB2-GFP or PR8/PB2-PspA virus showed an appreciable reduction in viral shedding in the lungs and nasal turbinates on days 3 and 6 after challenge, compared with those inoculated with medium (Table 1). These results show that immunization with the PR8/PB2-PspA virus conferred protective immunity against the lethal challenge with influenza virus.

Next, we assessed the protective efficacy of intranasal immunization with the PR8/PB2-PspA virus in a commonly used *S. pneumoniae* challenge model [20, 39, 46]. All mice mock-immunized with medium or immunized with the PR8/PB2-GFP virus died after the *S. pneumoniae* challenge (Figure 4*C*). By contrast, all mice immunized with the PR8/PB2-PspA virus were protected from the lethal challenge with *S. pneumoniae* (Figure 4*C*). These results demonstrate that the PR8/PB2-PspA virus conferred protective immunity against the lethal challenge with *S. pneumoniae*.

Table 1. Viral Titers in the Respiratory Tract of PR8/PB2-PspA Virus–Immunized Mice Challenged With PR8 Virus[a]

Immunization	Time After Challenge, d	Viral Titer, Mean (SD) $\log_{10}$ PFUs/g: Nasal Turbinates	Viral Titer, Mean (SD) $\log_{10}$ PFUs/g: Lungs
Medium	3	7.4 (0.2)	8.5 (0.4)
	6	7.0, NA, NA[b]	7.1, NA, NA[b]
PR8/PB2-GFP virus	3	6.9 (0.2)	7.8 (0.9)
	6	5.8 (0.3)	6.4, 4.5
PR8/PB2-PspA virus	3	7.8, 7.9	8.5
	6	ND[c]	ND

Abbreviations: GFP, green fluorescent protein; NA, not applicable; ND, not detected; PFUs, plaque-forming units; PspA, pneumococcal surface protein A; SD, standard deviation.

[a] Mice were intranasally immunized with the indicated agents (50 μL) 3 times at 2-week intervals. Two weeks after the final vaccination, the immunized mice were challenged with 10-fold 50% mouse lethal dose of PR8 virus (50 μL). Nasal turbinates and lungs were harvested from the challenged mice (n = 3) on days 3 and 6 after challenge and subjected to virus titration by plaque assays in AX4/PB2 cells. When virus was not recovered from all 3 mice, individual titers are provided.

[b] NA because the mice died.

[c] The detection limit was 1.5 $\log_{10}$ PFUs/g.

Effect of Intranasal Immunization With the PR8/PB2-PspA Virus on Secondary Pneumococcal Pneumonia

We next examined the protective efficacy of the PR8/PB2-PspA virus against secondary infection with a sublethal dose of *S. pneumoniae* after influenza virus infection [46]. The survival rate of mice immunized with the PR8/PB2-PspA virus (100%) was significantly higher than that of the PR8/PB2-GFP virus–immunized (50%) and the mock-immunized (20%) mice after secondary pneumococcal pneumonia (Figure 5*A*).

We also determined viral titers and bacterial densities in the respiratory organs of the mice subjected to secondary pneumococcal pneumonia after influenza virus infection. Viral titers in the lungs, but not those in the nasal turbinates, of mice immunized with either the PR8/PB2-GFP virus or the PR8/PB2-PspA virus were significantly lower than those in mice mock-immunized with medium (Table 2). The bacterial densities in mice immunized with either the PR8/PB2-GFP ($P < .05$) or the PR8/PB2-PspA virus ($P < .01$) were significantly lower than those in mock-immunized mice (Figure 5*B*). In addition, the bacterial densities in the lungs of the PR8/PB2-PspA virus–immunized mice were significantly lower than those in the PR8/PB2-GFP virus–immunized mice.

Finally, we examined the protective efficacy of the PR8/PB2-PspA virus against secondary infection with a lethal dose of *S. pneumoniae*. The survival rate of mice immunized with the PR8/PB2-PspA virus (100%) was significantly higher than that of the PR8/PB2-GFP virus–immunized (10%) or mock-immunized (0%) mice after secondary pneumococcal pneumonia ($P < .01$; Figure 5*C*). The bacterial density in the lungs of mice immunized with the PR8/PB2-PspA virus was significantly lower than that of the mock-immunized or the PR8/PB2-GFP virus–immunized mice ($P < .01$; Figure 5*D*). These results demonstrate that immunization with the PR8/PB2-PspA virus protects mice from secondary pneumococcal pneumonia.

DISCUSSION

In the current study, we generated a PB2-KO virus expressing the PspA of *S. pneumoniae* as a foreign protein in the coding region of its PB2 segment. We found no evidence of increased susceptibility of mice to pneumococcal pneumonia after intranasal inoculation of the PR8/PB2-PspA virus (Figure 2), indicating that our PB2-KO virus can be safely used in mice as a vaccine against *S. pneumoniae*.

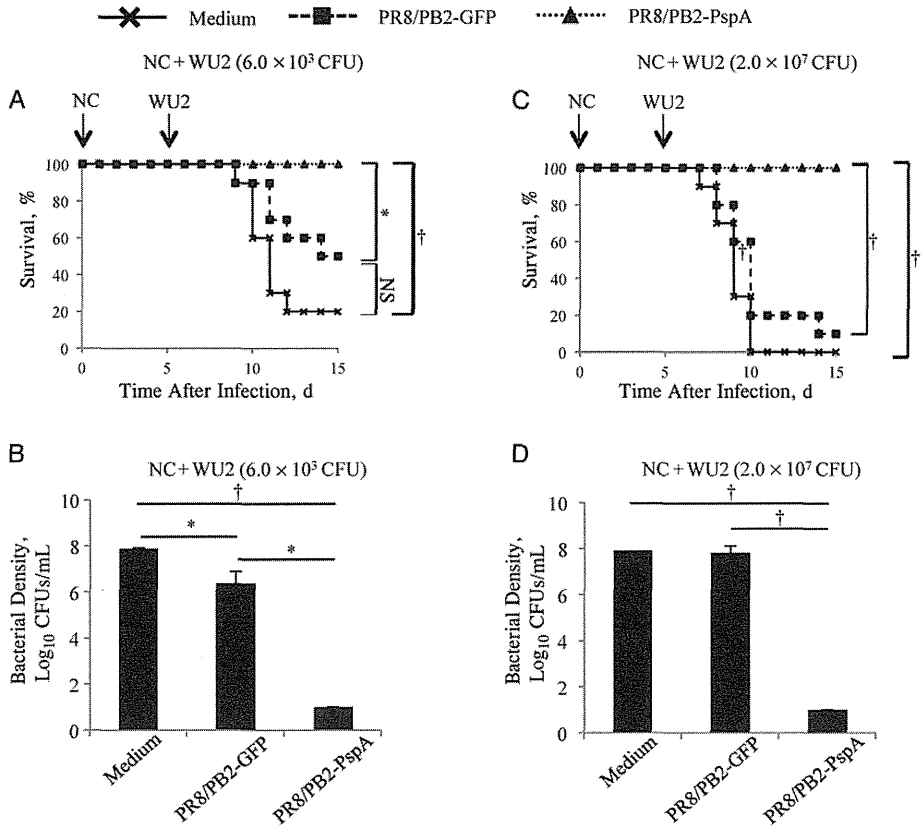

Figure 5. Survival of immunized mice after secondary pneumococcal pneumonia challenge and bacterial densities in mice. Mice were mock-immunized with medium or immunized with the PR8/PB2–green fluorescent protein (GFP) or PR8/PB2–pneumococcal surface protein A (PspA) virus 3 times at 2-week intervals. Two weeks after the final vaccination, mice were intranasally challenged with 1.0×10^3 plaque-forming units (PFUs) of A/New Caledonia/20/99 (NC) virus. Ten days after the viral challenge, mice were intranasally challenged with 6.0×10^3 colony-forming units (CFUs) (sublethal dose; *A, B*) or 2.0×10^7 CFUs (3-fold 50% mouse lethal dose; *C, D*). *A, C,* Survival was monitored in 10 mice for 10 days after the bacterial challenge. *$P < .05$; †$P < .01$. *B, D,* Lung tissues were harvested 5 days after bacterial challenge and subjected to bacterial titration on sheep blood agar. The detection limit of *Streptococcus pneumoniae* is 10 CFUs/mL. Values represent mean (standard deviation) $\log_{10}$ CFUs per lung for 6 mice per group. *$P < .05$; †$P < .01$. Abbreviation: NS, not statistically significant.

Intranasal immunization with the PR8/PB2-PspA virus could induce influenza virus–specific IgG in the airways and serum, and influenza virus–specific IgA in the airways of mice without the need for adjuvant (Figure 3*A* and 3*B*). We found that hemagglutination inhibition titers in mice immunized with the PR8/PB2-PspA virus were higher than those in PR8/PB2-GFP virus–immunized or mock-immunized mice (data not shown), suggesting that PspA might affect virus-specific immune responses. Similarly, intranasal immunization with the PR8/PB2-PspA virus could induce PspA-specific IgG in the airways and serum, and PspA-specific IgA in the lower airways of mice without the need for adjuvant (Figure 4*A* and 4*B*). We also demonstrated that intranasal immunization with 2.5 μg of PspA and 10 μg of a Toll-like receptor agonist were required to induce PspA-specific IgA in the airways [20]. Collectively, these results imply that the PR8/PB2-PspA virus may stimulate innate immunity, leading to the effective production of PspA-specific IgA in the airways.

The PR8/PB2-PspA virus elicited PR8-specific antibodies efficiently and protected mice from a lethal challenge with PR8 virus (Figure 3), as demonstrated elsewhere [35–37]. In addition, we found an inhibitory effect of intranasal immunization with PR8/PB2-KO virus on the replication of NC virus in the lungs of mice (Table 2). We also demonstrated the effect of intranasal immunization with PR8/PB2-PspA virus on bacterial clearance in the nasopharynx of mice.

The protective effect of intranasal immunization with PR8/PB2-PspA virus against pneumococcal pneumonia can be explained by the critical role of PspA-specific IgA for clearance in pneumococcal infection [49] and by the PspA-specific IgG-mediated opsonophagocytic killing activity [27, 50]. Importantly, we found no statistically significant difference in

Table 2. Viral Titers in the Respiratory Tract of PR8/PB2-PspA Virus–Immunized Mice Challenged With NC Virus[a]

Immunization	Viral Titer, Mean (SD), $\log_{10}$ PFUs/g	
	Nasal Turbinates	Lungs
Medium	6.3 (0.4)	6.1 (0.3)
PR8/PB2-GFP virus	6.0 (0.4)	4.0 (1.4)[b]
PR8/PB2-PspA virus	5.9 (0.4)	3.7 (1.3)[c]

Abbreviations: GFP, green fluorescent protein; NC, A/New Caledonia/20/99; PFUs, plaque-forming units; PspA, pneumococcal surface protein A; SD, standard deviation.

[a] Mice were intranasally immunized 3 times with the indicated agents (50 μL) and challenged with NC virus (50 μL) 2 weeks after the final vaccination. Nasal turbinates and lungs were harvested from mice (n = 6) 3 days after challenge and subjected to virus titration by use of plaque assays in AX4/PB2 cells. Statistically significant differences in the viral titers compared with the medium (control) group were assessed using 1-way analysis of variance followed by a Dunnett test.

[b] $P < .05$.

[c] $P < .01$.

protection, as measured by the survival rate after infection with a low-dose of *S. pneumoniae* (Figure 5*A*), for mice intranasally immunized with the PR8/PB2-GFP virus in the secondary infection model, although this was not the case with the high-dose challenge with *S. pneumoniae* (Figure 5*C*). Our data suggest that intranasal immunization with the PR8/PB2-GFP virus partly diminished the effect of preinfection with influenza virus on the enhanced susceptibility to pneumococcal infection. Of note, although intranasal immunization with the PR8/PB2-GFP virus did not protect the mice after infection with a high-dose of *S. pneumoniae*, all of the mice immunized with the PR8/PB2-PspA virus survived after infection with not only a low dose, but also a high dose of *S. pneumoniae* after influenza virus infection (Figure 5*A* and 5*C*). Taken together, these data suggest that the PR8/PB2-PspA virus is a powerful bivalent vaccine for both influenza virus and *S. pneumoniae* infections.

It is possible that PB2-KO vaccines may not induce immunity in those who have immunity to influenza viruses. Therefore, further studies are needed to examine whether the PR8/PB2-PspA virus can serve as a bivalent vaccine for mice previously infected with a homologous or heterologous influenza A virus strain. However, as the antigenicity of circulating influenza viruses changes, the backbone of the PB2-KO viruses could be replaced with that of the circulating strains.

In conclusion, in the current study we demonstrated the potential of a PB2-KO influenza virus expressing PspA from the recombinant PB2 gene as a novel bivalent vaccine against influenza virus and *S. pneumoniae*, respectively, but also against secondary pneumococcal pneumonia after influenza virus infection. Our PB2-KO virus therefore provides a promising option to control respiratory infections caused by common respiratory pathogens.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Nationwide population-based surveillance of invasive pneumococcal disease in Japanese children: Effects of the seven-valent pneumococcal conjugate vaccine

Shigeru Suga[a,*,1], Bin Chang[b,1], Kazutoyo Asada[a], Hideki Akeda[c], Junichiro Nishi[d], Kenji Okada[e], Hiroshi Wakiguchi[f], Akihiko Maeda[g], Megumi Oda[h], Naruhiko Ishiwada[i], Akihiko Saitoh[j], Tomohiro Oishi[j], Mitsuaki Hosoya[k], Takehiro Togashi[l], Kazunori Oishi[b], Toshiaki Ihara[a]

[a] *Infectious Disease Center and Department of Clinical Research, National Hospital Organization Mie Hospital, Mie, Japan*
[b] *National Institute of Infectious Diseases, Tokyo, Japan*
[c] *Okinawa Prefectural Nanbu Medical Center & Children's Medical Center, Okinawa, Japan*
[d] *Kagoshima University Graduate School of Medical and Dental Sciences, Kagoshima, Japan*
[e] *Section of Pediatrics, Department of Medicine, Division of Oral and Medical Management, Fukuoka Dental College, Fukuoka, Japan*
[f] *Kochi University, Kochi, Japan*
[g] *Department of Pediatrics, Kochi Prefectural Hata-Kenmin Hospital, Kochi, Japan*
[h] *Okayama University Graduate School of Health Sciences, Okayama, Japan*
[i] *Department of Infectious Diseases, Medical Mycology Research Center, Chiba University, Chiba, Japan*
[j] *Department of Pediatrics, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*
[k] *Department of Pediatrics, Fukushima Medical University School of Medicine, Fukushima, Japan*
[l] *Sapporo City University School of Nursing, Hokkaido, Japan*

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ABSTRACT

Background: In Japan, the seven-valent pneumococcal conjugate vaccine (PCV7) was introduced in 2010. PCV13 has replaced PCV7 since November 2013.
Methods: The effectiveness of PCV7 in protecting against invasive pneumococcal disease (IPD) in children aged <5 years was evaluated in a nationwide active population-based surveillance of IPD in 2008–2013 in 10 prefectures in Japan.
Results: 1181 cases were identified; 711 pneumococcal strains were analyzed for serotyping and antimicrobial resistance. Compared with the baseline IPD incidence (25.0 per 100,000), a 98% decline in IPD caused by PCV7 serotypes was found after the introduction of PCV7. This was partially offset by an increased incidence of IPD caused by PCV13 minus PCV7 and non-PCV13 serotypes, resulting in a 57% decline in overall IPD incidence. Absolute increases in the incidence rates of IPD caused by PCV13 minus PCV7 and non-PCV13 serotypes were 2.1 and 2.8 per 100,000 during the study period, respectively. The proportion of meropenem-nonsusceptible strains, especially with serotypes 19A and 15A, increased significantly after PCV7 introduction.
Conclusions: Our data confirmed a 98% decline in IPD incidence caused by PCV7 serotypes in children aged <5 years and serotype replacement after PCV7 introduction. This shows the importance of continuing surveillance of serotypes responsible for IPD and their antimicrobial resistance in Japan.

1. Introduction

Streptococcus pneumoniae is a major etiological pathogen of otitis media, pneumonia, bacteremia, and meningitis in children, and causes substantial morbidity and mortality worldwide [1–3]. Introduction of the seven-valent pneumococcal conjugate vaccine (PCV7) has markedly decreased the incidence of invasive pneumococcal disease (IPD) among vaccinated young children worldwide

Abbreviations: IPD, invasive pneumococcal disease; PCV, pneumococcal conjugate vaccine; CI, confidence interval.

* Corresponding author at: Infectious Disease Center and Department of Clinical Research, National Hospital Organization Mie Hospital, 357 Osatokubota, Tsu, Mie 514-0125, Japan. Tel.: +81 59 232 2531; fax: +81 59 232 5994.
E-mail address: sugas@mie-m.hosp.go.jp (S. Suga).
[1] These authors contributed equally to this article.

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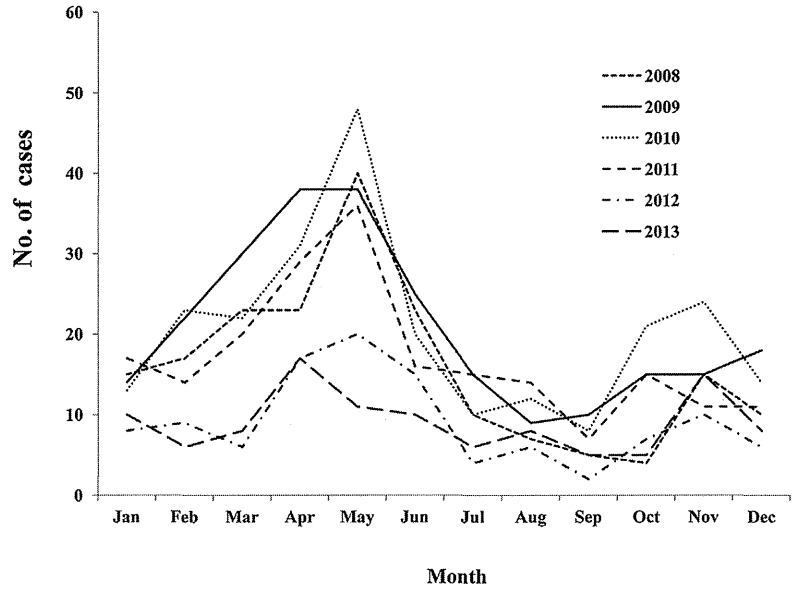

Fig. 1. Monthly incidence of invasive pneumococcal disease among children aged <5 years during the 6-year study period (2008–2013). The seven-valent pneumococcal conjugate vaccine (PCV7) was introduced in Japan on a voluntary basis among children aged <5 years in February 2010.

[4–7]. However, in the setting of broad-scale use of PCV7, previous studies have reported that non-PCV7-type IPD could increase in prevalence and reduce the benefits of vaccination, the so-called "serotype replacement" [5–14]. The active bacterial core surveillance system in the USA showed a continued decline in PCV7-type IPD incidence through 2007 (81.9 to 0.4, 99.5% decrease, among children aged <5 years) [14]. However, the study reported that further reductions in the overall incidence of IPD in 2002–2007 were partially offset by an increase in non-PCV7-type IPD, predominantly 19A (2.6 to 11.1, 324% increase, among children aged <5 years) [14]. A 13-valent PCV (PCV13), containing six additional serotypes, has been developed to cover a wider range of serotypes.

In Japan, PCV7 has been available on a voluntary basis since February 2010. From November 2010, PCV7 vaccination was further encouraged for children aged <5 years by an official program, the Provisional Special Fund for the Urgent Promotion of Vaccination by the Japanese government, resulting in an increased vaccination rate (89.2% in 2014) [Sakiyama H, Oishi K, unpublished data]. PCV7 has been included in the national immunization program since April 2013 and was completely replaced by PCV13 in November 2013.

Data from a prospective active surveillance study are essential to evaluating the effect of PCV7 introduction. However, to our knowledge, a nationwide population-based surveillance of IPD in children has never been performed in Japan. In the present study, we conducted a nationwide surveillance and analyzed the data collected from 10 prefectures in Japan. We used an active population-based system to evaluate the effects of PCV7 on IPD epidemiology, including changes in the incidence of IPD among children <5 years, serotype distributions, and antimicrobial resistance of pneumococcal isolates before and after the introduction of PCV7.

2. Materials and methods

2.1. Study design

Information about IPD cases involving children aged <5 years was collected in 10 prefectures (Supplementary Fig. 1) from January 2008 to December 2013. The under-5-year-old population of these 10 prefectures corresponded to about 23% of that of Japan. An IPD case was defined as the occurrence of IPD confirmed from a positive culture for *S. pneumoniae* in the blood, cerebrospinal fluid, or other fluid obtained from usually sterile sites. If a pediatrician treated a child with IPD who lived in that prefecture, the doctor notified the surveillance personnel using standardized case report forms. All hospitals that deliver pediatric care in each prefecture were actively contacted periodically for the identification of unreported cases. The data collected for these patients were sent to Mie Hospital. The isolated strains from normally sterile clinical samples were sent to the National Institute of Infectious Diseases for serotyping.

This study was reviewed and approved by the Ethics Committee of Mie Hospital and was conducted according to the principles expressed in the Declaration of Helsinki.

2.2. Serotyping

Serotypes of pneumococcal isolates from IPD patients were classified into three groups: PCV7 serotypes, PCV13 minus PCV7 serotypes, i.e., six additional serotypes to PCV7, and non-PCV13 serotypes. All of the isolates studied were serotyped using the Quellung reaction with pneumococcal antisera (Statens Serum Institut, Copenhagen, Denmark). Isolates were confirmed to be *S. pneumoniae* using a previously described method [15]. Isolates that did not react with any antiserum and those for which no obvious capsule was observed using Indian ink staining or no pneumococcal antigen was detected using a latex agglutination test (Slidex® Pneumo-Kit, BioMérieux, Marcy-l'Étoile, France) were classified as nontypable.

2.3. Antimicrobial susceptibility test

The MIC values of a total of 710 pneumococcal isolates were determined using the broth microdilution method according to a protocol of the Clinical and Laboratory Standards Institute (CLSI M100-S18). The antibiotics used were penicillin G, cefotaxime, meropenem, panipenem, and vancomycin. Antimicrobial susceptibility MIC breakpoints were defined according to the CLSI criteria [16]. We used the penicillin breakpoints for meningitis in