Arch Pharm Res Vol 30, No 3, 366-371, 2007

Archives of Pharmacal Research
http://apr.psk.or.kr

Intranasal Vaccination with Peptides and Cholera Toxin Subunit B as Adjuvant to Enhance Mucosal and Systemic Immunity to Respiratory Syncytial Virus

Hyoung Jin Kim, Jung-Kwon Kim*, Sang Byum Seo, Hoan Jong Lee[1], and Hong-Jin Kim

College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea and [1]College of Medicine, Seoul National University, Seoul 110-744, Korea

(Received November 27, 2006)

Amino acid sequences 200-225 and 255-278 of the F protein of human respiratory syncytial virus (HRSV) are T cell epitopes (Bourgeois *et al.*, 1991; Corvaisier *et al.*, 1993). Peptides corresponding to these two regions were synthesized and coupled with keyhole limpet haemocyanin (KLH). The two conjugated proteins were administered intranasally to BALB/c mice alone or together with cholera toxin B (CTB). ELISAs revealed that the mixture of the conjugates with CTB increased not only the systemic response but also the mucosal immune response of the saliva. The systemic response was lower and the mucosal immune response was undetectable in mice immunized with the conjugates on their own. These results suggest that these two peptide sequences are effective epitopes for inducing systemic and mucosal immune responses in conjunction with CTB, and may provide the basis for a nasal peptide vaccine against RSV for human use.

Key words: Respiratory syncytial virus, Mucosal immunization, Synthetic peptide

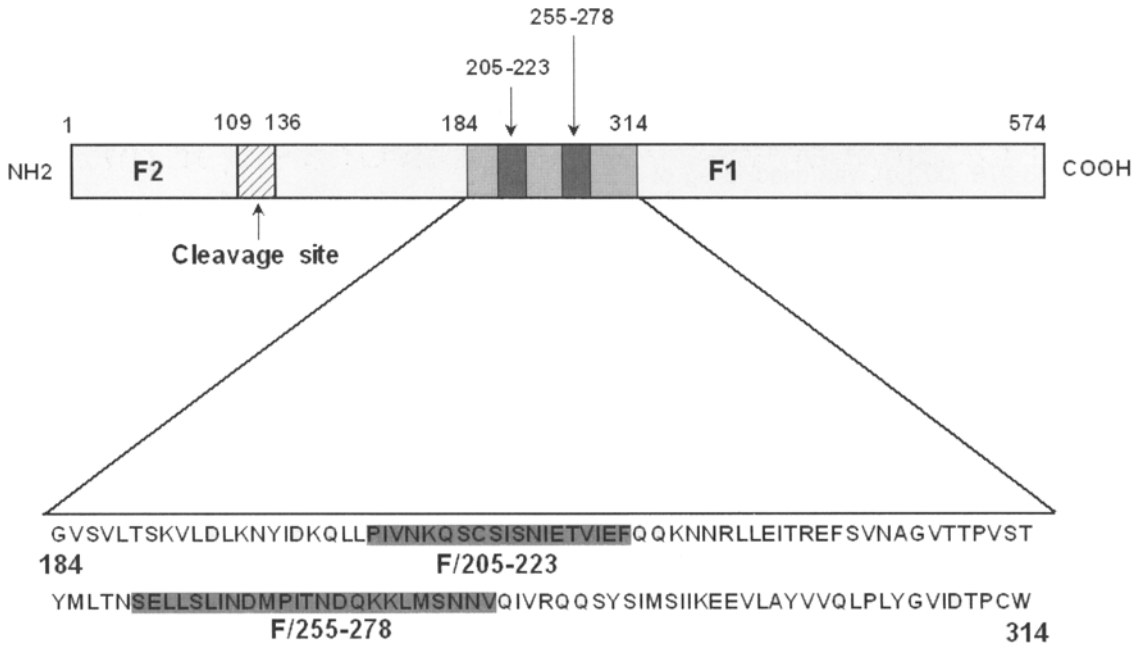
INTRODUCTION

Human Respiratory Syncytial Virus (HRSV) is a major respiratory pathogen responsible for respiratory tract disease in infants and immunodeficient patients (Goetsch *et al.*, 2001; Martinez-Sobrido *et al.*, 2006). It causes bronchiolitis and pneumonia, and approximately 85,000 infants and young children are hospitalized for it each year in the United State alone (Paramore *et al.*, 2004). Many pediatric viral diseases have been eradicated or reduced in severity by vaccination programs, but no safe and effective vaccine yet exists for RSV, despite the exploration of many approaches (Crowe. 2001; Martinez-Sobrido *et al.*, 2006). The most important characteristic of RSV is its ability to cause repeated infection in human because of great antigenic variability (Goetsch *et al.*, 2000). Clinical trials conducted in the 1960s using formalin-inactivated alum-precipitated whole RSV (FI-RSV) not only failed to protect against natural infection but also caused greatly enhanced symptoms in response to subsequent natural infection (Domachowske *et al.*, 1999). Although the exact mechanisms are not known, it is assumed that vaccination with FI-RSV disrupted the Th1/Th2 balance and increased the Th2 immune response (Tebbey *et al.*, 2000). Therefore, alternative immunization strategies are required (Bembridge *et al.*, 2000; Tebbey *et al.*, 2000; Piedra. 2003).

Most infectious agents enter the body via mucosal surfaces such as the respiratory and the gastrointestinal tract, and recent vaccine strategies have concentrated on blocking virus entry by producing mucosal antibodies (Stambas *et al.*, 2002). Mucosal IgA is expected to efficiently prevent RSV infection because it prevents the virus from binding to the mucosal surface (Bastien *et al.*, 1999).

RSV has two main surface glycoproteins in the viral membrane: the attachment (G) protein (Johnson *et al.*, 2004), which mediates binding to the cell receptor, and the fusion (F) protein, responsible for fusion of the viral and host cell membranes during entry (Techaarpornkul *et al.*, 2002). The F and G proteins on the surface of RSV are known to be important neutralization antigens as well

*Present address: Dong Kook R&D Center, 150-3, Hoejuk-ri, Kwanghaewon-myun, Jincheon-gun, Chungbuk, 365-834, Korea.
Correspondence to: Hong-Jin Kim, College of Pharmacy, Chung-Ang University, Seoul 156-756, Korea
Tel: 82-2-820-5613, Fax: 82-2-816-7338
E-mail: hongjink@cau.ac.kr



Scheme 1. The locations of F/205-223 and F/255-278 of the fusion protein of respiratory syncytial virus (RSV). A linear representation of the HRSV F precursor protein sequence. The numbers refer to residues from the N-terminus. The hatched box indicates the disulfide linkage between F1 and F2 subunits. The central boxed region encloses residues 184 to 314 of the F1 subunit that react with the neutralizing monoclonal antibody against RSV. The regions 205-223 and 255-278 induce T helper responses and these regions are represented as two dark box. Synthetic peptides F/205-223 and F/255-278 correspond to the regions 205-223 and 255-278 of the F1 subunit.

as major protective antigens (Johnson *et al.*, 2004). As shown in Scheme 1, the F protein is synthesized as an inactive precursor of 574 amino acids and has a molecular weight of 70 kDa (Gonzalez-Reyes *et al.*, 2001; Zimmer *et al.*, 2001). This precursor is cleaved at residues 109 136 by furin-like proteases in the *trans*-Golgi, generating the 50 kDa F1 and 20 kDa F2 polypeptides (Scheme 1) (Day *et al.*, 2006); F1 and F2 are linked by a disulfide bond (Day *et al.*, 2006).

Vaccination of BALB/c mice with the F protein induces strong pulmonary inflammation, because of Th2-driven lung eosinophilia and Th1-driven pulmonary infiltration (Chen *et al.*, 2002). However, vaccination with F protein induced both MHC-restricted CD8+ T lymphocytes and Th1 CD4+ T lymphocytes (Bembridge *et al.*, 2000). Hence the lung eosinophilia was not enhanced upon subsequent infection with RSV (Bembridge *et al.*, 2000). Residues 200-225 and 255-278 of the F sequence are known to induce rabbit serum neutralizing RSV (Bourgeois *et al.*, 1991; Corvaisier *et al.*, 1993, 1997). In addition, peptide 255-278 enhanced the T cell response to subsequent immunization with F protein *in vitro* (Corvaisier *et al.*, 1993). However, there is no report of the effect of immunizing BALB/c mice intranasally with both of these peptides.

In this study we synthesized two peptides corresponding to amino acids 205 to 223 and 255 to 278 of the F protein and coupled them with keyhole limpet haemocyanin (KLH) (Scheme 1). We report that mice generate both mucosal and systemic immune responses following intranasal vaccination with the two peptides in combination with cholera toxin B.

MATERIALS AND METHODS

Mice

Female BALB/c (Charles river) mice aged 5 weeks were purchased from Orientbio Company (Kyonggi, Korea). They were used after adaptation for seven days in conditions of 12 h light and dark, and constant temperature (21~23°C) and humidity (55%).

Synthesis and coupling of peptides

Peptides F/205-223 (PIVNKQSCSISNIETVIEF) and F/255-278 (SELLSLINDMPITNDQKKLMSNNV) corresponding to amino acid sequences 205-223 and 255-278 of the F1 subunit of HRSV F protein (Scheme 1) were produced by solid phase synthesis. They were dissolved in DMSO at 20 mg/mL (Herd *et al.*, 2006) and coupled to KLH (Fischer *et al.*, 2003).

Immunization

Groups of five BALB/c mice were anesthetized intraperitoneally (i.p.) with ketamine (60 mg/kg, Yuhan Corp.,

Korea) (Tebbey *et al.*, 2000) and immunized on days 0, 14 and 28 by intranasal (i.n) administration of F/205-223 or F/255-278. Each mouse was immunized with 60 μg of peptide F/205-223 or F/255-278. When both peptides were administered, a total of 60 μg of peptides F/205-223 (30 μg) and F/255-278 (30 μg) was used. 5 μg of CTB was administered to each mouse. 20 μg of KLH and 5 μg of CTB were administered to mice of the control group (Table I, Table II).

Collection of samples

After first immunization, serum and saliva were obtained on days 21, 35, 42. Blood samples were drawn and sera were obtained by centrifugation. Saliva were collected by washing with 50 μL of phosphate buffered saline (PBS), using a micropipette. Serum and saliva samples were stored at -20°C.

Table I. The serum antibody response in mice immunized intranasally with peptides F/205-225 and F/255-278

Antigens	ELISA titer[a] (log_{10})		
	IgG		
	21 days	35 days	42 days
F/205-223 (60 μg)	2.6±0.5	3.2±0.4	2.2±1.3
F/205-223 (60 μg) + CTB[b] (5 μg)	4.4±0.1	4.3±0.1	4.4±0.1
F/255-278 (60 μg)	3.3±0.2	2.2±0.3	2.3±0.4
F/255-278 (60 μg) + CTB (5 μg)	4.1±0.3	4.2±0.2	4.3±0.1
F/205-223 (30 μg) + F/255-278 (30 μg)	3.7±0.1	2.7±0.7	2.1±1.2
F/205-223 (30 μg) + F/255-278 (30 μg) + CTB (5 μg)	4.2±0.5	4.5	4.3±0.1
KLH (20 μg) + CTB (5 μg)	< 2	< 2	< 2

[a]Data are presented as log_{10} of the endpoint titer that resulted in an OD 405. End point titers were deduced at an optical density (OD) of 1.5 times background. Data are representative of four independent experiments in which similar results were obtained. ($P < 0.0005$)
[b]CTB represents the cholera toxin B.

Table II. The mucosal antibody response in mice immunized intranasally with peptides F/205-225 and F/255-278

Antigens	ELISA titer[a] (log_{10})		
	IgA		
	21 days	35 days	42 days
F/205-223 (60 μg)	< 1.3	< 1.3	< 1.3
F/205-223 (60 μg[1]) + CTB[b] (5 μg)	1.4±0.2	2.2±0.2	2.0±0.2
F/255-278 (60 μg)	< 1.3	< 1.3	< 1.3
F/255-278 (60 μg) + CTB (5 μg)	1.7± 0.2	1.7±0.1	1.4±0.1
F/205-223 (30 μg) + F/255-278 (30 μg)	< 1.3	< 1.3	< 1.3
F/205-223 (30 μg) + F/255-278 (30 μg) + CTB (5 μg)	1.6±0.2	2.3 ±0.1	2.0±0.2
KLH (20 μg) + CTB (5 μg)	< 1.3	< 1.3	< 1.3

[a]Data are presented as log_{10} of the endpoint titer that resulted in an OD 492. End point titers were deduced at an optical density (OD) of 1.5 times background. Data are representative of four independent experiments in which similar results were obtained. ($P < 0.05$)
[b]CTB represents the cholera toxin B.

Titration of peptide-specific antibodies

Peptide-specific antibodies were detected by indirect ELISA as described previously (Fleming *et al.*, 1988). KLH-conjugated F/205-223 and F/255-278 were diluted with carbonate buffer pH 9.1. Ninety-six-well microtitre plates (Greiner Bio-one, Germany) were coated overnight at 4°C with 100 ng of KLH-conjugated F/205-223 and F/255-278 per well. When the group of mice that had received both peptides was titrated, the plates were coated with a total of 100 ng of F/205-223 (50 ng) and F/255-278 (50 ng). The plates were blocked with 2% BSA in phosphate buffer pH 7.4 for 30 min at room temperature. Serum and saliva samples were serially two-fold diluted with diluent buffer (0.6% BSA in phosphate buffer pH 7.4) and aliquots were incubated in the wells for 1 h at room temperature. After washing the wells three times with wash solution (2 mM imidazole. 0.9% NaCl, 0.02% tween 20), 100 μL of HRP-conjugated anti-mouse IgG (0.5 μg/mL) (Kirkegaard & Perry Laboratories Inc., U.S.A) and HRP-conjugated anti-mouse IgA (0.3 μg/mL) (Sigma, U.S.A.) were added to the wells and incubated for 1 h. After washing five times, 100 μL of ABTS (PIERCE, U.S.A) and o-phenylenediamine (Sigma, U.S.A.) were added to the wells. OD 405 nm and OD 492 nm values were measured to detect peptide-specific serum IgG and saliva IgA, respectively. End-point titers were deduced at an optical density (OD) of 1.5 times background (Bastien *et al.*, 1999).

Statistical analysis

The statistical differences between all vaccinated goups and the control (KLH + CTB administed group) were determined by the paired *t*-test of the SigmaPlot software (SPSS Inc., U.S.A.) on the log_{10} titre. Differences with $P < 0.0005$ and $P < 0.05$ were considered significant.

RESULTS

Humoral immune responses of mice immunized with F/205-223 and F/255-278

Scheme 1 illustrates that the amino acid sequence from 184-Gly to 314-Trp of the F1 subunit reacts with neutralizing monoclonal antibodies, and that the sequences 205-223 and 255-278 contain the T cell epitopes (Bourgeois *et al.*, 1991; Corvaisier *et al.*, 1993).

After mice were vaccinated intranasally with F/205-223 and F/255-278, sera were obtained on days 21, 35, 42, and peptide-specific serum IgG was measured by indirect

ELISA. In addition, we assessed whether the presence of cholera toxin B (CTB) as a mucosal adjuvant improved the immune responses induced by the peptides. The log titer of the control group, which was administered with KLH and CTB, was less than 2.0. Therefore, KLH and CTB did not affect the stimulation of peptide-specific serum IgG. When F/205-223 and/or F/255-278 were administered in the absence of CTB, the highest log titer of peptide-specific serum IgG obtained with F/205-223 was 3.2 on day 35, and the highest log titers obtained with F/255-278 and the two peptides together were 3.3 and 3.7, respectively, on day 21 (Table I). Although the maximum log titers of these groups were over 3.0, they were not sustained up to day 42 in the absence of CTB (Table I). When the peptides were administered in the presence of CTB, the log titers of peptide-specific serum IgG for F/205-223 and F/255-278 and for the co-administered group exceeded 4.0 by the time of the prime boost on day 21 and these titers were maintained until day 42 (Table I). Therefore, CTB accelerated the humoral immune responses and resulted in their levels being maintained. Evidently the two peptide sequences induce an effective humoral immune response in the presence of CTB.

Mucosal immunity after immunization with F/205-223 and F/255-278

After mice were vaccinated intranasally with F/205-223 and F/255-278, we obtained mouth wash samples on days 21, 35, 42 and measured peptide-specific saliva IgA by indirect ELISA. The log titer of the control group administered KLH and CTB was less than 1.3. Therefore, KLH and CTB did not affect the stimulation of peptide-specific saliva IgA. When F/205-223 and/or F/255-278 were administered in the absence of CTB, the log titers of peptide-specific saliva IgA were less than 1.3 (Table II). This indicates that the peptides were unable to induce a mucosal immune response in the absence of CTB. When F/205-223 and/or F/255-278 were administered along with CTB, the log titers of these groups were maximal on day 35 and decreased by day 42. The log titer of F/205-223 was 2.2, and those of the mice receiving F/255-278 and both peptides were 1.7 and 2.3, respectively, on day 35 (Table II). Therefore, peptide F/205-223 was more effective than F/255-278 in inducing salivary IgA and the similarity of the results obtained with F/205-223 and the two peptides together seems that F/205-223 affect the titer in the presence of CTB (Table II). These results indicate that CTB is an essential adjuvant for the mucosal immune response.

DISCUSSION

The intranasal route is a comparatively easy route of antigen delivery to mucosal surfaces, avoiding the requirement for passage through the acidic stomach barrier (Bastien *et al.*, 1999). Mucosal immunization offers several advantages over parenteral immunization, including greater efficacy in achieving both mucosal and systemic antibody responses and minimal adverse reactions. It also reduces the danger of cross-contamination with needles (Bastien *et al.*, 1999; Goetsch *et al.*, 2001).

In previous reports, The chimeric FG glycoprotein consists of amino acids 1 to 489 from RSV F followed by amino acids 97 to 279 from RSV G was adminstered intransally to BALB/c mice and induced serum IgG and mucosal IgA against RSV (Oien *et al.*, 1994). However, in the reports of Tebby *et al.* (Tebbey *et al.*, 1998) and Sparer *et al.*(Sparer *et al.*, 1998), amino acids sequence from position 183 to 197 of RSV G protein induce lung eosiniphilia. The RSV chimeric FG glycoprotein contains the sequence of G protein (Wathen *et al.*, 1989). For this reason, RSV chimeric FG seems to cause eosinophilia upon live RSV challenge in cotton rats (Connors *et al.*, 1992). Therefore, recent formulations of candidate RSV have focused syntheitc peptide vaccine that induce a selective immune reponses and minimize adverse effects (Corvaisier *et al.*, 1993; Piedra. 2003). However, no reports of mucosal immune responses based on synthetic peptides have been reported. Therefore, we investigated humoral and mucosal immune responses when synthetic peptides F/205-223 and F/255-278 mixed with CTB were administered intranasally to BALB/c mice. The ELISA titers (log 10) of peptide-specific serum IgG were more than 4.0, and those of peptide-specific saliva IgA were between 1.7 and 2.2 in the presence of CTB, whereas background titers of saliva IgA were found in the absence of CTB (Table I, II). Therefore, CTB was shown to be an essential adjuvant for inducing the mucosal immune response. However, peptide specific serum IgG were induced in the absence of CTB. This result distinguished the result of Goetsch *et al.* (Goetsch *et al.*, 2001). In that experiment, BBG2Na (residues 130-230 of RSV G protein fused to BB, an albumin binding domain of streptococcal protein G) cannot induce anti-G2Na serum and saliva antibodies in the absence of CTB (Goetsch *et al.*, 2001). This result indicates the amino acid sequence 205-223 and 255-278 of F1 subunit are effective sequences to induce T-helper response. In addition, unlike previous reports using 10 μg/dose (Goetsch *et al.*, 2001; Isaka *et al.*, 2001), CTB was administetered at 5 μg/dose. Therefore, higher titers of antibodies against F/205-223 and F/255-278 are expected at 10 μg/dose.

In the report of Bastien *et al.*, synthetic peptides based on the RSV G protein together with CTB, titers of serum IgG were high while those of saliva IgA were background (Bastien *et al.*, 1999). It seems that the cause of these

difference lies in the T-helper response (Marinaro *et al.*, 1998), which is affected by the nature of the peptide sequence. In our case, the stimulatory effects of the two peptides on salivary IgA in the presence of CTB differed: F/205-223 induced peptide-specific salivary IgA more efficiently than F/255-278 (Table II). Bourgeois *et al.* (1991) reported that peptides corresponding to amino-acids 200-225 and 255-278 of F protein are T-cell epitopes, and they emphasized that peptide 200-225 is the immune dominant region of the F protein, which induced the development of a rabbit anti-peptide serum neutralizing RSV *in vitro* (Bourgeois *et al.*, 1991). This result agrees with our finding that F/205-223 induced peptide-specific saliva IgA more efficiently than F/255-278.

We have shown that high levels of peptide-specific serum IgG and saliva IgA were induced when two synthetic peptides for T cell epitopes were administered intranasally. This suggests that peptides 205-223 and 255-278 may be used an effective nasal spray vaccine. We are currently investigating how the serum IgG and saliva IgA generated in response to peptides 205-223 and 255-278 neutralize RSV, and whether pulmonary damage results from vaccination with these peptides.

REFERENCES

Bastien, N., Trudel, M., and Simard, C., Complete protection of mice from respiratory syncytial virus infection following mucosal delivery of synthetic peptide vaccines. *Vaccine*, 17, 832-836 (1999).

Bembridge, G. P., Rodriguez, N., Garcia-Beato, R., Nicolson, C., Melero, J. A., and Taylor, G., Respiratory syncytial virus infection of gene gun vaccinated mice induces Th2-driven pulmonary eosinophilia even in the absence of sensitisation to the fusion (F) or attachment (G) protein. *Vaccine*, 19, 1038-1046 (2000).

Bourgeois, C., Corvaisier, C., Bour, J. B., Kohli, E., and Pothier, P., Use of synthetic peptides to locate neutralizing antigenic domains on the fusion protein of respiratory syncytial virus. *J. Gen. Virol.*, 72 (Pt 5), 1051-1058 (1991).

Chen, M., Hu, K. F., Rozell, B., Orvell, C., Morein, B., and Liljestrom, P., Vaccination with recombinant alphavirus or immune-stimulating complex antigen against respiratory syncytial virus. *J. Immunol.*, 169, 3208-3216 (2002).

Connors, M., Collins, P. L., Firestone, C. Y., Sotnikov, A. V., Waitze, A., Davis, A. R., Hung, P. P., Chanock, R. M., and Murphy, B. R., Cotton rats previously immunized with a chimeric RSV FG glycoprotein develop enhanced pulmonary pathology when infected with RSV, a phenomenon not encountered following immunization with vaccinia--RSV recombinants or RSV. *Vaccine*, 10, 475-484 (1992).

Corvaisier, C., Bourgeois, C., and Pothier, P., Cross-reactive and group-specific immune responses to a neutralizing epitope of the human respiratory syncytial virus fusion protein. *Arch. Virol.*, 142, 1073-1086 (1997).

Corvaisier, C., Guillemin, G., Bourgeois, C., Bour, J. B., Kohli, E., and Pothier, P., Identification of T-cell epitopes adjacent to neutralizing antigenic domains on the fusion protein of respiratory syncytial virus. *Res. Virol.*, 144, 141-150 (1993).

Crowe, J. E. Jr., Respiratory syncytial virus vaccine development. *Vaccine*, 20 Suppl 1, S32-37 (2001).

Day, N. D., Branigan, P. J., Liu, C., Gutshall, L. L., Luo, J., Melero, J. A., Sarisky, R. T., and Del Vecchio, A. M., Contribution of cysteine residues in the extracellular domain of the F protein of human respiratory syncytial virus to its function. *Virol. J.*, 3, 34 (2006).

Domachowske, J. B. and Rosenberg, H. F., Respiratory syncytial virus infection: immune response, immunopathogenesis, and treatment. *Clin. Microbiol. Rev.*, 12, 298-309 (1999).

Fischer, D., Rood, D., Barrette, R. W., Zuwallack, A., Kramer, E., Brown, F., and Silbart, L. K., Intranasal immunization of guinea pigs with an immunodominant foot-and-mouth disease virus peptide conjugate induces mucosal and humoral antibodies and protection against challenge. *J. Virol.*, 77, 7486-91 (2003).

Fleming, J. O. and Pen, L. B., Measurement of the concentration of murine IgG monoclonal antibody in hybridoma supernatants and ascites in absolute units by sensitive and reliable enzyme-linked immunosorbent assays (ELISA). *J. Immunol. Methods*, 110, 11-18 (1988).

Goetsch, L., Gonzalez, A., Plotnicky-Gilquin, H., Haeuw, J. F., Aubry, J. P., Beck, A., Bonnefoy, J. Y., and Corvaia, N., Targeting of nasal mucosa-associated antigen-presenting cells in vivo with an outer membrane protein A derived from Klebsiella pneumoniae. *Infect. Immun*, 69, 6434-6444 (2001).

Goetsch, L., Plotnicky-Gilquin, H., Aubry, J. P., De-Lys, P., Haeuw, J. F., Bonnefoy, J. Y., Nguyen, N. T., Corvaia, N., and Velin, D., BBG2Na an RSV subunit vaccine candidate intramuscularly injected to human confers protection against viral challenge after nasal immunization in mice. *Vaccine*, 19, 4036-4042 (2001).

Goetsch, L., Plotnicky-Gilquin, H., Champion, T., Beck, A., Corvaia, N., Stahl, S., Bonnefoy, J. Y., Nguyen, T. N., and Power, U. F., Influence of administration dose and route on the immunogenicity and protective efficacy of BBG2Na, a recombinant respiratory syncytial virus subunit vaccine candidate. *Vaccine*, 18, 2735-2742 (2000).

Gonzalez-Reyes, L., Ruiz-Arguello, M. B., Garcia-Barreno, B., Calder, L., Lopez, J. A., Albar, J. P., Skehel, J. J., Wiley, D. C., and Melero, J. A., Cleavage of the human respiratory syncytial virus fusion protein at two distinct sites is required for activation of membrane fusion. *Proc. Natl. Acad. Sci. U.S.A.*, 98, 9859-9864 (2001).

Herd, K. A., Mahalingam, S., Mackay, I. M., Nissen, M., Sloots, T. P., and Tindle, R. W., Cytotoxic T-lymphocyte epitope vaccination protects against human metapneumovirus

infection and disease in mice. *J. Virol*, 80, 2034-2044 (2006).

Isaka, M., Yasuda, Y., Mizokami, M., Kozuka, S., Taniguchi, T., Matano, K., Maeyama, J., Mizuno, K., Morokuma, K., Ohkuma, K., Goto, N., and Tochikubo, K., Mucosal immunization against hepatitis B virus by intranasal co-administration of recombinant hepatitis B surface antigen and recombinant cholera toxin B subunit as an adjuvant. *Vaccine*, 19, 1460-1466 (2001).

Johnson, T. R., Teng, M. N., Collins, P. L., and Graham, B. S., Respiratory syncytial virus (RSV) G glycoprotein is not necessary for vaccine-enhanced disease induced by immunization with formalin-inactivated RSV. *J. Virol.*, 78, 6024-6032 (2004).

Marinaro, M., Boyaka, P. N., Kiyono, H., and McGhee, J. R., Novel approaches for the induction of T helper 1 (Th1)- or Th2-type mucosal and parenteral immune responses. *Expert. Opin. Investig Drugs*, 7, 1657-1666 (1998).

Martinez-Sobrido, L., Gitiban, N., Fernandez-Sesma, A., Cros, J., Mertz, S. E., Jewell, N. A., Hammond, S., Flano, E., Durbin, R. K., Garcia-Sastre, A., and Durbin, J. E., Protection against respiratory syncytial virus by a recombinant Newcastle disease virus vector. *J. Virol.*, 80, 1130-1139 (2006).

Oien, N. L., Brideau, R. J., Walsh, E. E., and Wathen, M. W., Induction of local and systemic immunity against human respiratory syncytial virus using a chimeric FG glycoprotein and cholera toxin B subunit. *Vaccine*, 12, 731-735 (1994).

Paramore, L. C., Ciuryla, V., Ciesla, G., and Liu, L., Economic impact of respiratory syncytial virus-related illness in the US: an analysis of national databases. *Pharmacoeconomics*, 22, 275-284 (2004).

Piedra, P. A., Clinical experience with respiratory syncytial virus vaccines. *Pediatr. Infect. Dis. J.*, 22, S94-9 (2003).

Sparer, T. E., Matthews, S., Hussell, T., Rae, A. J., Garcia-Barreno, B., Melero, J. A., and Openshaw, P. J., Eliminating a region of respiratory syncytial virus attachment protein allows induction of protective immunity without vaccine-enhanced lung eosinophilia. *J. Exp. Med.*, 187, 1921-1926 (1998).

Stambas, J., Pietersz, G., McKenzie, I., and Cheers, C., Oxidised mannan as a novel adjuvant inducing mucosal IgA production. *Vaccine*, 20, 1068-1078 (2002).

Tebbey, P. W., Hagen, M., and Hancock, G. E., Atypical pulmonary eosinophilia is mediated by a specific amino acid sequence of the attachment (G) protein of respiratory syncytial virus. *J. Exp. Med.*, 188, 1967-1972 (1998).

Tebbey, P. W., Scheuer, C. A., Peek, J. A., Zhu, D., LaPierre, N. A., Green, B. A., Phillips, E. D., Ibraghimov, A. R., Eldridge, J. H., and Hancock, G. E., Effective mucosal immunization against respiratory syncytial virus using purified F protein and a genetically detoxified cholera holotoxin, CT-E29H. *Vaccine*, 18, 2723-2734 (2000).

Techaarpornkul, S., Collins, P. L., and Peeples, M. E., Respiratory syncytial virus with the fusion protein as its only viral glycoprotein is less dependent on cellular glycosaminoglycans for attachment than complete virus. *Virology*, 294, 296-304 (2002).

Wathen, M. W., Brideau, R. J., Thomsen, D. R., and Murphy, B. R., Characterization of a novel human respiratory syncytial virus chimeric FG glycoprotein expressed using a baculovirus vector. *J. Gen. Virol.*, 70 (Pt 10), 2625-2635 (1989).

Zimmer, G., Budz, L., and Herrler, G., Proteolytic activation of respiratory syncytial virus fusion protein. Cleavage at two furin consensus sequences. *J. Biol. Chem.*, 276, 31642-50 (2001).