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[Review]

Neutralizing epitopes of RSV and palivizumab resistance in Japan

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Abstract

Respiratory Syncytial Virus (RSV) is one of the most important viral pathogen related to acute lower respiratory infection in young children. The virus surface envelope contains the G, F, and SH proteins as spike proteins. The F protein is considered to be a major antigenic target for the neutralizing (NT) epitope as only the F protein is essential for cell infection among the three viral envelope proteins, and it is more highly conserved than the G protein. Recently, four antigenic targets related to NT activity have been reported ; site I, site II, site IV, and site zero (0). Site II is the target for palivizumab used throughout the world to suppress severe RSV infection as passive immunity in high-risk children since 1998. Under the recent conditions in which indications for palivizumab administered subjects are being expanded, palivizumab-resistant mutations have been confirmed overseas in children with RSV infection, although they remain infrequent. Therefore, continuous genetic analysis of the palivizumab-binding region of the F protein is necessary. In addition, as vaccine development progresses, RSV infection control is expected to improve greatly over the next decade.

Key words : RSV, neutralizing epitope, palivizumab, resistance, Japan

Introduction

Respiratory Syncytial Virus (RSV) is one of the most important viral pathogen related to acute lower respiratory infection (ALRI) in young children[1]. RSV infection accounts for 1/3 of deaths from ALRI in infants aged under 1 year and is the second largest cause of death from infectious diseases worldwide after malaria, with up to 200 thousand children, mainly in developing countries, losing their lives due to RSV infection each year[2]. In addition, the severity of infection in the high-risk group[3,4] as well as the onset of airway hyperresponsiveness/asthma after RSV infection are problems[5,6]. Patients with RSV infection usually receive symptomatic treatment, including oxygen administration, fluid replacement, and respiratory management[7]. RSV monoclonal antibodies have been administered to high-risk children as a prophylactic measure[8]. RSV monoclonal antibodies have been administered to high-risk children as a prophylactic measure, on the other hand, there are several ongoing trials in preclinical, Phase-I, Phase-II, or Phase-III clinical stages for RSV vaccine development[9]. Analysis of the RSV epitope is important for the development of more effective RSV monoclonal antibodies and vaccines.

Virological structure

RSV is a single-stranded (−) RNA virus classified as a member of the genus Orthopneumovirus in the family Pneumoviridae, and is composed of 10 genes including NS1, NS2 encoding nonstructural protein, N encoding nucleoprotein, P encoding phosphoprotein, M/M2 encoding matrix protein, SH encoding small hydrophobic protein (SH protein), G encoding large glycoprotein (G protein), F encoding fusion protein (F protein), and L encoding large polymerase protein. The virus surface envelope contains the G, F, and SH proteins as spike proteins. RSV is classified into two subgroups, A and B, due

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to differences in reactivity with monoclonal antibodies to the G protein[10].

F protein

The F protein of RSV is a 574 amino acids class I fusion glycoprotein similar to influenza virus hemagglutinin and HIV-1 envelope glycoprotein[11,12]. It is synthesized as a precursor F_0 and cleaved by the furin-like host protease to produce F_1 and F_2 subunits[13,14]. The mature protein contains three copies of two polypeptides (F_1 and F_2). After initially folding into a metastable prefusion conformation (pre-F), RSV F undergoes a structural change naturally or in the process of infection, and eventually acquires a stable postfusion conformation (post-F)[12]. Recently, cryoelectron tomography of cell culture-grown RSV has revealed that pre-F and post-F are present on the virion surface. In addition, The pre-F and post-F are about 11 nm and 16 nm high, with the distribution dependent on the age and state of the virus[15,16].

Neutralizing epitope

Of the 11 proteins that comprise RSV, only the F and G proteins are targeted by neutralizing antibodies[17]. The F protein is considered to be a major antigenic target for the neutralizing (NT) epitope as only the F protein among the three viral envelope proteins is essential for cell infection, and F protein is more highly conserved than G protein[18]. Four antigenic targets related to NT activity have been reported. Site I is an antigenic target for monoclonal antibodies (mAbs), such as 2F, 44F, or 45F[19], with weak or negligible NT activity. Site II is recognized by murine 47F and 1129[20,21], with humanization of the latter resulting in palivizumab[22] and its more potent derivative motavizumab[23]. Palivizumab is licensed and widely used throughout the world to suppress severe RSV infection in high-risk children[24]. Site IV is recognized by mAb 19[25] or 101 F[26] with moderate NT activity. A recently isolated human mAb, 54G10, shows NT activity for human metapneumovirus (hMPV), which belongs to genus paramyxovirus as RSV[27]. The 54G10 mAb is predicted to recognize antigenic site IV. Of these three epitopes, site I exists only in post-F, and site II and site IV exist in both pre-F and post-F. All the mAbs that recognize these three sites can bind to the stable post-F conformation[28]. Studies using RSVIG, an immunoglobulin product enriched for high RSV NT activity, showed that adsorbing the product with post-F did not remove the NT activity

Table 1. Conformation of the F protein and epitopes

Conformation	Stability	Epitope	Ab(s)[a]	Remarks regarding Ab[a]
Pre-F [c]	metastable	site 0 (zero)	D25, AM22, 5C4	✓ 10- to 100-fold more potent than palivizumab
		site II	murine 47F, 1129	✓ Mouse monoclonal antibody that was the basis of palivizumab development.
			palivizumab	✓ a licensed mAb administered prophylactically to infants at high risk of severe RSV-related disease
			motavizumab	✓ a more potent derivative of palivizumab
		site IV	101F, Mab19	✓ moderate NT activity[b]
			54G10	✓ neutralizes hMPV and RSV
		adjacent to site II	MPE8	✓ cross-competes with palivizumab ✓ neutralizes multiple pneumoviruses in the Paramyxoviridae
Post-F [c]	stable	site I	2F, 44F, 45F	✓ weak or negligible NT activity[b]
		site II	47F, 1129 palivizumab motavizumab	(same as Pre-F remarks)
		site IV	101F, Mab19	(same as Pre-F remarks)
			54G10	(same as Pre-F remarks)

a) Ab(s) shows antibody or antibodies.
b) NT activity means neutralizing activity.
c) Pre-F, and Post-F indicate prefusion conformation F, and post-fusion conformation F, respectively.

from the immunoglobulins elicited by natural infection[17], suggesting that there are other NT-sensitive targets on the pre-F. Site zero (0) was recently revealed to exist in the pre-F as the major fourth antigenic target[28]. Monoclonal antibodies against site 0 include D25, AM22, and 5C4, all of which are prefusion-specific and have NT activity 10-100 fold greater than that of palivizumab[28,29]. MPE8 is an additional prefusion-specific antibody that which cross-competes with palivizumab for binding to pre-F[30]. MPE8 can neutralize not only human and bovine RSV, but also hMPV and pneumonia virus in mice, suggesting that the MPE8 epitope is particularly well-conserved among pneumoviruses[30] (Table 1). Based on the recently revealed characteristics of neutralizing epitope against RSV, not only new monoclonal Abs but also vaccines as the final goal of overcoming infectious diseases are expected in the future.

Palivizumab

For use in providing passive immunity to RSV infection, a humanized monoclonal antibody palivizumab (Synagis®) targeting the antigenic site II (also called site A) region of the RSV F protein has been developed by genetic recombination technology[22,31] and is now used throughout the world. There are many antigenic variations in the G protein among subtypes, whereas there are few for the F protein. Therefore, an antibody targeting the F protein was created. On the other hand, the G protein is the most variable RSV protein, making it useful for RSV evolution studies[12]. Palivizumab has been approved and used in the United States since 1998 with the aim of suppressing the severity of RSV infection in high-risk children, such as preterm infants, and those with congenital heart disease or immunodeficiency[32]. It is currently used in more than 60 countries and has been shown to be effective in preventing severe infection in the high-risk group and subsequently alleviating airway hyperresponsiveness and asthma episodes[33-35].

The indications for palivizumab administration of USA is different from that of Japan. The American Academy of Pediatrics guidance for palivizumab use has become more restrictive against otherwise healthy preterm infants born at or after 29 weeks of gestation[36]. In Japan, palivizumab was listed as a prescribed drug from 2002, with the subjects for administration being preterm infants (newborns and infants under 12 months of age with preterm birth from 29 weeks to 35 weeks or newborns and infants under 6 months of age with premature birth of 28 weeks or less of gestation), neonates, infants and young children under 24 months of age who have received treatment for bronchopulmonary dysplasia within the past 6 months. In 2005, palivizumab dosing indications were added for newborns, infants and young children with congenital heart disease with abnormal hemodynamics below 24 months of age. Furthermore, indications for neonates, infants and young children with immunodeficiency or Down's syndrome less than 24 months of age were added in 2013[37-39].

Palivizumab resistance

Recently, there have been reports on the detection of viruses showing palivizumab neutralizing-resistant amino acid mutations. Clinical isolates N262D[40], K272E/M/Q[41,42], and S275F/L[40,42,43] were reported as possessing mutations in the F protein region palivizumab-binding site (amino acids 258-275) and exhibiting resistance to palivizumab neutralization, while laboratory induced isolates N268I, and K272N/T/M/Q have also been reported[43-46]. The N276S mutation adjacent to the palivizumab-

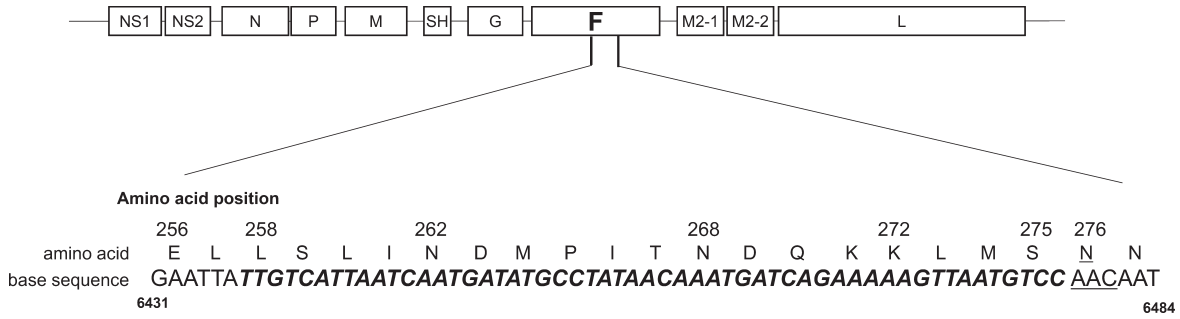

Fig. 1. RSV genome and palivizumab-binding site
Amino acids (aa) and base sequences are shown based on the Long strain, which is a standard RSV-A strain. Bold and italic characters indicate palivizumab-binding site, aa 258-275. Mutations at aa 276 (underlined) have been increasing in recent years.

binding site in RSV-A was confirmed in Israel and Turkey in the 2007-2008 season[40] and was dominant in Canada between 2008 and 2010[41]. Adams *et al*. showed that the N276S mutation in RSV-A leads to a decrease in NT activity against palivizumab[47]. This mutant virus was isolated from preterm infants 4 months old who were administered palivizumab but were treated in an intensive care unit for RSV infection. Furthermore, when the isolate virus was cultured in the presence of palivizumab, a second amino acid mutation, K272E, occurred in the palivizumab-binding site abolishing its ability to bind to palivizumab. On the other hand, Papenburg *et al*. conducted F gene analysis of 23 RSV strains isolated from patients with palivizumab and RSV 100 strains isolated from children without palivizumab during the 4 seasons from 2006 to 2010[41]. The N276S mutation was detected in RSV-A at a rate of 44.4% in 2008-2009 and 100% in 2009-2010. Furthermore, they reported that the N276S mutation was observed in children both with and without a history of palivizumab treatment[41]. In addition, Zhu *et al*. demonstrated that the N276S mutation in RSV-A and the S276N mutation in RSV-B did not result in palivizumab neutralization resistance based on neutralization tests using clinical isolates and recombinant virus[40]. However, in a mouse experiment, mutation of the 276th amino acid in the F protein was shown to induce MARMs (Monoclonal Antibody-Resistant Mutants) showing resistance to the mouse monoclonal antibody against the antigenic site II region[20,44], indicating that the 276th amino acid is susceptible to selection pressure by the antibody.

Zhu *et al*. investigated natural gene polymorphisms involving the F protein in RSV isolates from patients with respiratory disease without palivizumab treatment[40,43]. The detection frequency of palivizumab neutralization resistant mutations was 0.7%, and these mutations were reported to be random mutations that occurred due to selective pressure by maternal or the patient's own NT antibodies against site II of the RSV F protein. On the other hand, they also reported that the detection frequency of palivizumab-resistant mutations in RSV isolated from patients receiving palivizumab treatment was 5.4%[43]. The amino acid at position 276 of the F protein is generally asparagine (N) for RSV-A and serine (S) for RSV-B[40,47]. It is possible that the N276S mutation in RSV-A and S276N mutation in RSV-B appeared due to hybridization between the subgroups, natural mutation and so on. However, since the N276S mutation has not been reported in RSV isolated before the use of palivizumab, the predominance of the N276S mutation may be related to some selective pressure attributable to palivizumab[20,44]. Since the palivizumab binding ability is completely lost when a second amino acid mutation is introduced following the N276S mutation, the N276S mutation is considered to be the first amino acid mutation inducing palivizumab neutralization resistance.

Recently, the ON1 strain of RSV-A[48,49] and BA strain of RSV-B[50] have spread to various parts of the world over several seasons. These strains possess mutations in the G protein associated with adsorption to host cells, indicating that the immunogenicity of the G protein is involved in the epidemic expansion. On the other hand, the N276S mutation is a mutation in the F protein. The global dominance of the N276S mutation in the short term is thought to be due to some factors positively affecting virus growth. Viruses having palivizumab neutralization resistance mutations (K272 E/Q and S275 F/L) have a lower proliferative capacity than do wild type strains[40]. It is necessary to investigate the relation of virus proliferation ability and infectivity to amino acid mutations in segments other than antibody recognition segments, such as the N276S mutation.

The situation in Japan

Apart from our previous report[51], there have been no reports on palivizumab resistance in Japan. We analyzed palivizumab resistance using clinical isolates or PCR products derived from 116 airway secretion specimens from patients diagnosed with RSV infection in Miyagi prefecture between 2004 and 2008, and in Fukushima prefecture between 2008 and 2013. Only one RSV patient in 2013 received prophylaxis with palivizumab. The nucleotide sequence and amino acid sequence of amino acids 215-313 including the F protein region palivizumab-binding site were analyzed for all 116 strain. A comparison of the RSV-A and RSV-A Long strain revealed 23 silent mutations and 9 mutations (N228I/T, E236A, V239I, L258I, N276S, V281L, R282S, S290P, and D310H). A comparison of the RSV-B and RSV-B 9320 strain revealed 35 silent mutations and 4 mutations (S228N/T, L247V, S276N, and I305L). Among the detected amino acid mutations, the only mutation in the palivizumab-binding site was the L258I mutation in RSV-A, which was detected from a specimen from a patient not receiving palivizumab. The frequency of muta-

Table 2-1. Mutations and detection rates in the F protein of RSV-A

RSV-A (n=106)	
Amino acid mutation[a)]	Number of mutants (%)[b)]
228 (Asn → Ile)	1 (0.9)
228 (Asn → Thr)	2 (1.9)
236 (Glu → Ala)	6 (5.7)
239 (Val → Ile)	1 (0.9)
258 (Leu → Ile)	2 (1.9)
276 (Asn → Ser)	87 (82.1)
281 (Val → Leu)	1 (0.9)
282 (Arg → Ser)	1 (0.9)
290 (Ser → Pro)	3 (2.8)
310 (Asp → His)	3 (2.8)

a) Amino acid mutations were identified in comparison with the Long strain (Accession No. AY911262) of RSV-A.
b) The detection frequency of mutants was obtained by dividing the number of mutant strains by the total number of strains analyzed.
c) The palivizumab-binding site is in region of amino acid 258-275.
d) The table was cited from the reference 51.

Table 2-2. Mutations and detection rates in the F protein of RSV-B

RSV-B (n=10)	
Amino acid mutation[a)]	Number of mutants (%)[b)]
228 (Ser → Asn)	9 (90)
228 (Ser → Thr)	1 (10)
236 (Glu → Ala)	2 (20)
247 (Leu → Val)	10 (100)
276 (Ser → Asn)	5 (50)
305 (Ile → Leu)	10 (100)

a) Amino acid mutations were identified in comparison with the 9320 strain (Accession No. AY353550) of RSV-B.
b) The detection frequency of mutants was obtained by dividing the number of mutant strains by the total number of strains analyzed.
c) The palivizumab-binding site is in region of amino acid 258-275.
d) The table was cited from the reference 51.

tions other than N276S in RSV-A was 6% or less (0.9 to 5.7%). The frequency of N276S was 82.1% (82 out of 106 strains) in RSV-A (Table 2-1), and the frequency of the S276N mutation in RSV-B was 50% (5 out of 10 strains) (Table 2-2). The frequency of theN276S mutation in RSV-A is 0% (0/2) in 2004, 0% (0/3) in 2005, 0% (0/3) in 2006, 60% (3/5) in 2007, 22.2% (2/9) in 2009, 95.6% (22/23) in 2009, 96.8% (31/32) in 2010, 100% (4/4) in 2011, 100% (7/7) in 2012, and 100% (18/18) in 2013. In other words, the N276S mutation began to appear around 2007-2008 and was found in over 90% of the analyzed strains after 2009 (Table 3). Thus, the results in Japan are similar to those reported overseas[40]. Furthermore, the K272E mutation as second mutation was not reported in Japan and was not observed in our analysis. Analysis in children with RSV infection administered with palivizumab is necessary in the future.

Table 3. Annual change in N276S mutations in RSV-A

Year	Number of N276S mutations (%)[a)]
2004	0/2 (0)
2005	0/3 (0)
2006	0/3 (0)
2007	3/5 (60.0)
2008	2/9 (22.2)
2009	22/23 (95.6)
2010	31/32 (96.8)
2011	4/4 (100)
2012	7/7 (100)
2013	18/18 (100)

a) One-hundred and sixteen RSV clinical strains or PCR products of RSV-A were analyzed to detect N276S. The detection frequency for N276S was obtained by dividing the number of strains with N276S by the total number of strains analyzed in each year.
b) The table was cited from the reference 51 with slight modifications.

Conclusion

Under the recent conditions in which indications for palivizumab administration are being expanded, palivizumab-resistant mutations have been confirmed overseas in children with RSV infection, although they remain infrequent at present. In Japan, the N276S mutation thought likely to lead to palivizumab neutralization resistance is becoming dominant. Therefore, continuous genetic analysis of the F protein palivizumab-binding region is necessary. On the other hand, site 0, which leads to strong NT activity was also identified, and the development of monoclonal antibodies with site 0 as an epitope is certain to advance in the future. Vaccine development is also progressing, so that the control of RSV infection is expected to change markedly over the next decade.

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Conflicts of interest

The authors have no conflict of interest to disclose with respect to this manuscript.

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