Synergistic Pathogenic Effects of Combined Mouse Monoclonal Anti-Desmoglein 3 IgG Antibodies on Pemphigus Vulgaris Blister Formation

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Pemphigus vulgaris (PV) is an autoimmune blistering disease caused by anti-desmoglein 3 (Dsg3) IgG antibodies. Previously, we generated an active mouse model for PV by adoptive transfer of splenocytes from immunized or naive Dsg3$^{-/-}$ mice. In this study, we isolated 10 anti-Dsg3 IgG mAbs (NAK-series) from PV model mice generated by transfer of naive Dsg3$^{-/-}$ splenocytes. We characterized their epitopes using domain-swapped and point-mutated Dsg1/Dsg3 molecules and examined their pathogenic activities in blister formation in three different assays. In a passive transfer model using neonatal mice, eight of 10 NAK mAbs showed pathogenic activity when injected together with half the minimum pathogenic dose of anti-Dsg1 IgG autoantibodies from pemphigus foliaceus (PF) patients. None of the mAbs could induce the PV phenotype when individual hybridoma clones were inoculated by peritoneal injection into adult Rag2$^{-/-}$ mice. NAK mAbs displayed a range of potency in an *in vitro* dissociation assay using primary cultured mouse keratinocytes. Interestingly, when multiple hybridoma clones recognizing different epitopes were inoculated in combination, recipient mice developed the PV phenotype. *In vitro* dissociation assays confirmed that combined NAK mAbs had synergistic pathogenic effects. These findings indicate that although an individual anti-Dsg3 IgG is not sufficient to cause blistering in adult mice, several together can induce the PV phenotype. These mAbs will provide a valuable tool to investigate the molecular mechanisms of blister formation, mimicking the effects of the polyclonal IgG antibodies found in patients.

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INTRODUCTION

Pemphigus vulgaris (PV) is a fatal autoimmune blistering disease of the skin and mucous membranes (Amagai, 2003), which is characterized clinically by flaccid blisters and erosions, and histopathologically by the loss of cell–cell adhesion between basal and suprabasal keratinocytes, resulting in suprabasal acantholysis. The target antigen of PV is desmoglein 3 (Dsg3), a desmosomal transmembrane glycoprotein that belongs to the cadherin gene superfamily of Ca^{2+}-dependent cell–cell adhesion molecules (Amagai *et al.*, 1991; Amagai, 1996). Compelling evidence indicates that IgG autoantibodies against Dsg3 in PV play a primary pathogenic role in blister formation (Anhalt *et al.*, 1982; Amagai *et al.*, 1992, 1998; Mahoney *et al.*, 1999).

Previously, we developed a mouse model for PV using Dsg3$^{-/-}$ mice, which should not acquire immunological tolerance to Dsg3 (Amagai *et al.*, 2000b). After immunization of Dsg3$^{-/-}$ mice with recombinant extracellular domains of mouse Dsg3, we adoptively transferred splenocytes from the immunized mice into Rag2$^{-/-}$ recipient mice that express Dsg3. The transferred Dsg3$^{-/-}$ lymphocytes stably produced anti-Dsg3 IgG and the recipient mice developed a PV phenotype, including oral erosions with histological suprabasilar acantholysis and telogen hair loss (Koch *et al.*, 1997). Subsequently, we demonstrated that the adoptive transfer of naive splenocytes from nonimmunized Dsg3$^{-/-}$ mice to Rag2$^{-/-}$ recipients also induced the production of anti-Dsg3 IgG and the PV phenotype (Aoki-Ota *et al.*, 2004). Antibody production and the appearance of the PV phenotype were delayed by approximately 2 weeks in mice receiving naive splenocytes compared to those receiving immunized splenocytes. However, once the PV phenotype developed, there were no apparent differences in disease severity between mice induced by the two methods. Interestingly, the anti-Dsg3 IgG titers were markedly lower in mice that received naive splenocytes than in mice that received immunized

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Abbreviations: Dsg, desmoglein; hDsg, human desmoglein; mDsg, mouse desmoglein; PF, pemphigus foliaceus; PV, pemphigus vulgaris

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splenocytes, suggesting that the antibodies generated in the former are more potent than those in the latter. Conformational epitope mapping in model mice revealed that anti-Dsg3 IgG were predominantly raised against the middle to C-terminal extracellular domains of mouse Dsg3, where amino-acid residues are less conserved among desmoglein isoforms. PV model mice receiving naive splenocytes produced antibodies against the N-terminal domain of Dsg3 more frequently than mice receiving immunized splenocytes (Anzai *et al.*, 2004).

We isolated eight anti-Dsg3 IgG mAbs from PV model mice that received immunized Dsg3$^{-/-}$ splenocytes (the AK series) (Tsunoda *et al.*, 2003). Two of these mAbs were capable of inducing the loss of cell–cell adhesion in keratinocytes, as determined in a passive transfer model using neonatal mice, and one of the two, AK23, was able to induce the PV phenotype when hybridoma cells were inoculated into the peritoneal cavities of Rag2$^{-/-}$ mice. The other six mAbs failed to show apparent pathogenic activity, in spite of their ability to bind the native Dsg3 *in vivo*.

In the present study, we isolated a further 10 anti-Dsg3 IgG mAbs from PV model mice that received naive Dsg3$^{-/-}$ splenocytes (NAK series). We characterized the pathogenic activities of these mAbs in three different assays (passive transfer, ascites formation, and *in vitro* dissociation), and also analyzed their conformational epitopes. We found that anti-Dsg3 mAbs reacting with different parts of the molecule are synergistic in their pathogenic activity, inducing blister formation when they are combined.

RESULTS

Production of NAK anti-Dsg3 IgG mAbs from PV model mice

As a source of hybridoma cells, we used splenocytes of PV model mice generated by adoptive transfer of splenocytes from naive Dsg3$^{-/-}$ mice, which produce more potent anti-Dsg3 IgG antibodies than mice receiving immunized Dsg3$^{-/-}$ splenocytes (Aoki-Ota *et al.*, 2004). We first screened the hybridoma cells by ELISA against recombinant mouse Dsg3; positive clones were further screened by live staining of mouse keratinocyte PAM212 cells. The second screening selected mAbs that could bind to the native Dsg3 on keratinocyte cell surfaces *in vivo*. Ten independent clones were isolated and designated as NAK mAbs (Table 1).

All of the NAK mAbs had the κ isotype light chain. Four (NAK1, 2, 3, 5) had IgG1 and six (NAK4, 7, 8, 9, 10, 11) had IgG2a heavy chains (Table 1). Under indirect immunofluorescence, all stained keratinocyte cell surfaces in all layers of the mouse oral mucosa, with stronger intensity toward the more basal layers (Figure 1, mouse hard palate and see also Supplementary Figure 1) and in the basal and parabasal layers of the mouse epidermis (Figure 1, mouse tail skin). Five mAbs (NAK1, 4, 7, 8, 9) also stained the keratinocyte cell surfaces of human epidermis, but with different staining patterns. NAK1 and NAK9 mAbs stained the lower layers of the human epidermis, NAK4 stained all layers, and NAK7 and NAK8 stained the middle to upper layers (Figure 1, human epidermis). With ELISA, all of the mAbs reacted only with mouse Dsg3 and not with mouse Dsg1. Those mAbs that showed crossreactivity with human epidermis

Table 1. Characterization of the NAK mAbs

		IIF[1]		ELISA							Pathogenic activity		
				Mouse		Human[2]							
NAK	Isotype	Mouse	Human	Dsg3	Dsg1	Dsg3	Dsg1	Live keratinocyte staining	Ca dependency[3]	Epitopes[4]	Passive transfer[5]	Ascites formation	Dissociation assay[6]
1	IgG1κ	+	+	+	–	+	–	+	+	T25, Y28, Q29	+	–	14.6
2	IgG1κ	+	–	+	–	–	–	+	+	195–402	+	–	25.2
3	IgG1κ	+	–	+	–	+	–	+	–	403–565	–	–	0.5
4	IgG2aκ	+	+	+	–	+	+	+	+	1–162	+	–	35.9
5	IgG1κ	+	–	+	–	–	–	+	–	195–402	–	–	12.5
7	IgG2aκ	+	+	+	–	–	+	+	+	1–162	+	–	13.4
8	IgG2aκ	+	+	+	–	–	+	+	+	1–162	+	–	25.4
9	IgG2aκ	+	+	+	–	+	–	+	+	T25, Y28, Q29, V53 D54, K55, N56	+	–	45.1
10	IgG2aκ	+	–	+	–	–	–	+	+	1–162	+	–	34.9
11	IgG2aκ	+	–	+	–	–	–	+	+	1–402	+	–	33.6

IIF, indirect immunofluorescence.
[1]Indirect immunofluorescence staining. Mouse oral mucosa (hard palate) and human normal skin were used as the substrates.
[2]ELISA reactivity against human Dsgs was determined with 0.1 μg/ml NAK mAbs.
[3]The reactivity of NAK mAbs against mouse Dsg3 was determined with or without EDTA treatment. +, indicates the abolition of the reactivity by EDTA.
[4]The epitopes are indicated by the residue numbers for mouse Dsg3 except those for NAK1 and NAK9 mAbs, whose epitopes are indicated by the residues for human Dsg3.
[5]+, indicates gross blister formation when coinjected with PF IgG.
[6]Dissociation index values are listed when dissociation index obtained by AK23 mAb (Tsunoda *et al.*, 2003) is 100.

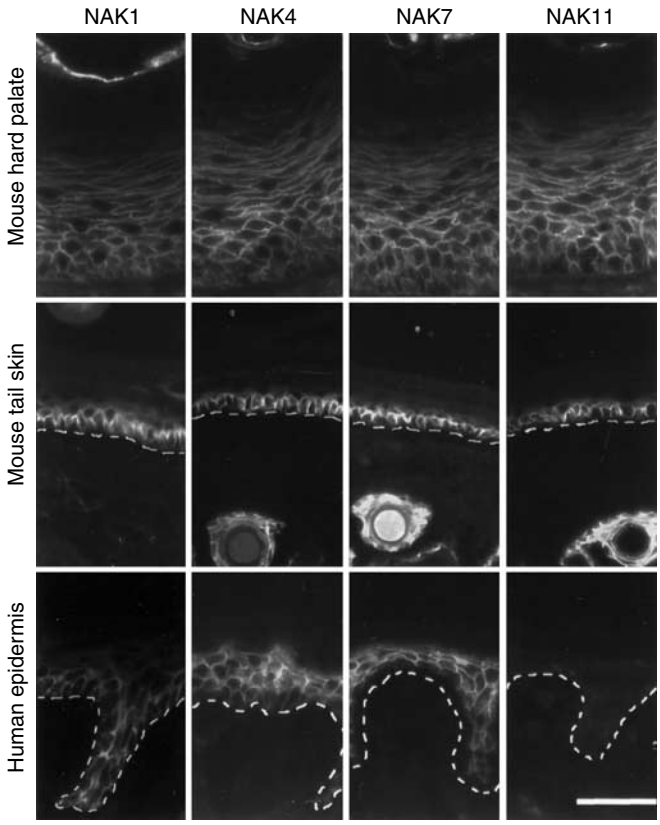

Figure 1. Immunostaining of mouse and human tissues with NAK mAbs. Representative immunostaining is shown (NAK1, NAK4, NAK7, and NAK11). All of the NAK mAbs stained keratinocyte cell surfaces in all layers of mouse oral mucosa (mouse hard palate), with stronger intensity toward the lower layers, and stained the lower layers of mouse epidermis (mouse tail skin), where Dsg3 is expressed. NAK mAbs reacted with human epidermis in different patterns (human epidermis); NAK1, NAK4, and NAK7 mAbs stained the lower portion of, the entire, and the upper portion of human epidermis, respectively, while NAK11 failed to react with human epidermis. Dashed lines indicate the basement membrane zone. Bar = 50 μm.

under immunofluorescence also recognized human Dsg3 and/or Dsg1 in different patterns. NAK1, NAK3, and NAK9 reacted with human Dsg3, NAK4 reacted with both human Dsg3 and Dsg1, and NAK7 and NAK8 reacted with human Dsg1 (Table 1). The immunofluorescence staining patterns of NAK mAbs whose IIF was positive on human epidermis (NAK1, 4, 7, 8, 9) were consistent with the ELISA results, based on the distribution of Dsg1 and Dsg3 in the human epidermis (Amagai *et al.*, 1996; Shirakata *et al.*, 1998).

Thus, all NAK mAbs specifically reacted with native mouse Dsg3 without crossreacting with mouse Dsg1, and some crossreacted with human Dsg3 and/or Dsg1 in different patterns.

NAK mAbs recognize different epitopes on Dsg3

To characterize the epitopes of the NAK mAbs, we determined whether their binding to mouse Dsg3 was dependent on calcium. Treating a mouse Dsg3-coated ELISA plate with EDTA did not substantially alter binding of NAK3 and NAK5, but abolished binding of the other mAbs (data not shown). Therefore, NAK3 and NAK5 mAbs recognized Ca^{2+}-independent epitopes, while the other mAbs recognized Ca^{2+}-dependent conformational epitopes.

To map the epitopes of the mAbs, we used domain-swapped mouse Dsg3/Dsg1 recombinant molecules (Anzai *et al.*, 2004). These molecules are believed to maintain native conformations, at least in terms of binding anti-Dsg IgG antibodies (Futei *et al.*, 2000; Sekiguchi *et al.*, 2001). We used four domain-swapped molecules that contained the mouse Dsg3 residues 1–162 ($mDsg3^{1–162}$/$mDsg1^{163–512}$), 1–402 ($mDsg3^{1–402}$/$mDsg1^{403–512}$), 195–565 ($mDsg1^{1–194}$/$mDsg3^{195–565}$), or 403–565 ($mDsg1^{1–402}$/$mDsg3^{403–565}$). NAK1, 4, 7, 8, 9, 10 mAbs precipitated residues 1–162 and 1–402, but not residues 195–565 or 403–565 (Figure 2a and b). Their epitopes appear to reside in residues 1–162, the N-terminal portion of the extracellular domain of mouse Dsg3. NAK2 and NAK5 reacted with residues 1–402 and 195–565, but not with residues 1–162 or 403–565 (Figure 2a and b). Their epitopes appear to be in residues 195–402, which represent the middle portion of the extracellular domain. NAK3 precipitated residues 195–565 and 403–565, but not residues 1–162 or 1–402 (Figure 2a and b). Its epitope appears to reside in residues 403–565, the C-terminal portion of the extracellular domain. NAK11 mAb precipitated only residues 1–402 (Figure 2a and b).

Of these mAbs, NAK1 and NAK9 crossreacted with human Dsg3 but not with human Dsg1, and recognized the N-terminal residues 1–162. We used a series of point-mutated human Dsg3 and Dsg1 molecules, in which Dsg3-specific residues were replaced by the corresponding Dsg1-specific residues, or *vice versa* (Figure 2d; Tsunoda *et al.*, 2003). NAK1 mAb maintained its reaction with human Dsg3 mutated at T31, K33, I34 (Dsg3-M2), V53-N56 (Dsg3-M3), E70, S73 (Dsg3-M4), L75, T77 (Dsg3-M5), A83, and Q84 (Dsg3-M6), but lost reactivity when the Dsg3 specific residues T25, Y28, or Q29 were mutated (Dsg3-M1, M1-2, M1-2-3) (Figure 2c and d). NAK1 gained recognition of human Dsg1 modified with Dsg3-specific residues at T25, Y28, or Q29 (Dsg1-M1-2, M1-2-3) (Figure 2c and d). These findings indicate that the epitope for NAK1 maps to the T25, Y28, and Q29 of Dsg3. T25 and Q29 are conserved between human and mouse Dsg3, but Y28 in the human is replaced by F28 in the mouse.

Similarly, NAK9 mAb reacted with human Dsg3 mutated at Dsg3-specific residues T31, K33, I34 (Dsg3-M2), E70, S73 (Dsg3-M4), L75, T77 (Dsg3-M5), A83, and Q84 (Dsg3-M6), but lost recognition when either T25, Y28, Q29 (M1), or V53-N56 (Dsg3-M3) were mutated (Figure 2c and d). NAK9 gained recognition of human Dsg1 when mouse residues T25, Y28, Q29, and V53-N56 were introduced (Dsg1-M1-2-3), but not when T25, Y28, and Q29 were introduced with T31, K33, and I34 (Dsg1-M1-2) (Figure 2c and d). These findings indicate that the epitope of NAK9 mAb maps to the residues T25, Y28, Q29, V53, D54, K55, and N56. Of these, Y28 and K55 are not conserved in mouse Dsg3 (F28, P55).

Thus, these NAK mAbs recognize different epitopes on Dsg3, and six of them recognize the N-terminal 1–162 residues (Table 1).

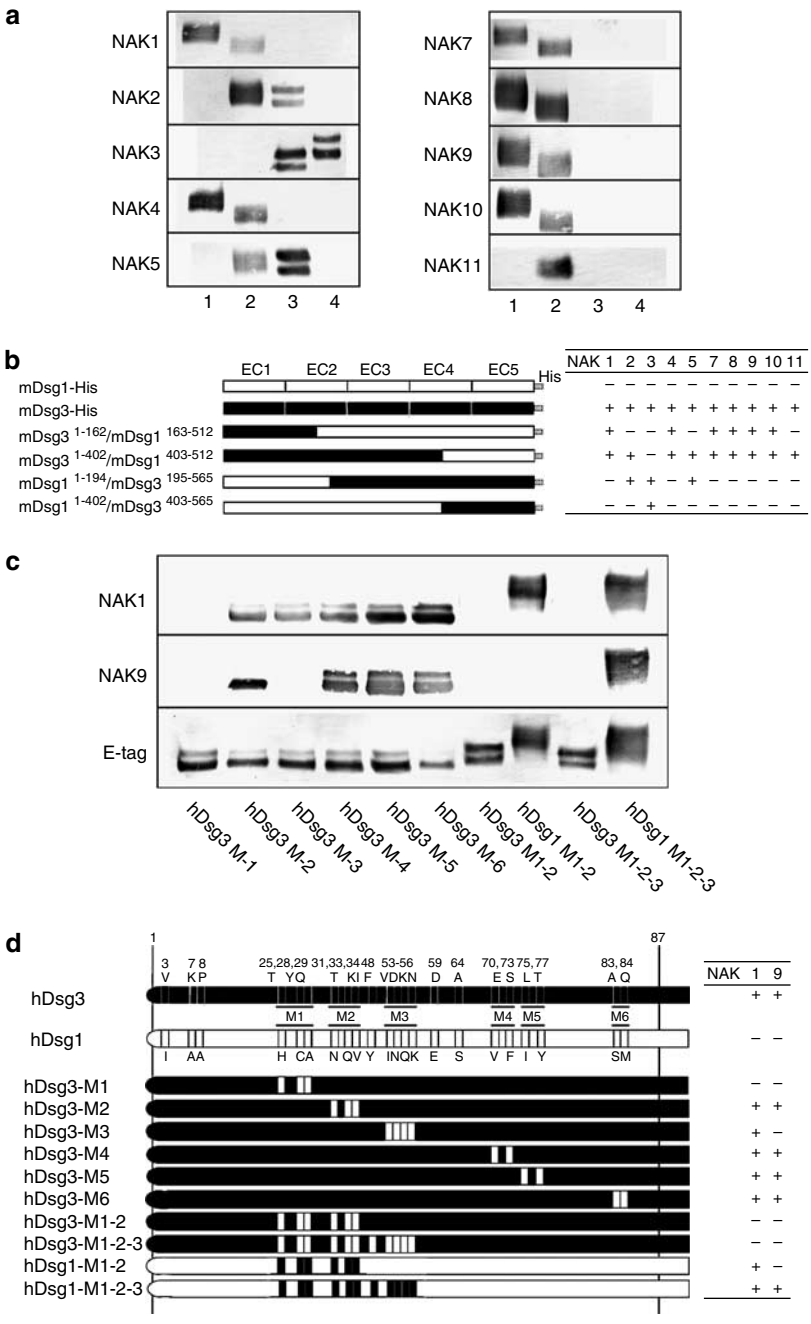

Figure 2. Immunoprecipitation of domain-swapped and point-mutated Dsg1/Dsg3 recombinant proteins with NAK mAbs. (**a**) Immunoprecipitation of mDsg3$^{1–162}$/mDsg1$^{163–512}$ (lane 1), mDsg3$^{1–402}$/mDsg1$^{403–512}$ (lane 2), mDsg1$^{1–194}$/mDsg3$^{195–596}$ (lane 3), and mDsg1$^{1–402}$/mDsg3$^{403–565}$ (lane 4) with various NAK mAbs. (**b**) The molecular structure of domain-swapped mDsg1/mDsg3 proteins and summary of results for epitope mapping of mAbs. (**c**) Immunoprecipitation of various point-mutated hDsg1/hDsg3 proteins with NAK1 (upper panel), NAK9 (middle panel), and anti-E-tag mAb as a positive control (lower panel). (**d**) The molecular structure of point-mutated hDsg1/hDsg3 proteins and summary of results for epitope mapping of NAK1 and NAK9 mAbs. The 22 amino-acid residues not conserved between hDsg1 and hDsg3 are indicated. These nonconserved residues were switched between Dsg1 and Dsg3 to generate point-mutated molecules.

Most NAK mAbs induce blisters in passive transfer to neonatal mice

To evaluate the pathogenic activities of the mAbs, we first performed a passive transfer assay using neonatal mice, a well-established assay for pemphigus (Anhalt *et al.*, 1982; Amagai *et al.*, 1994). Purified IgG from the culture supernatant of each hybridoma was injected s.c. into neonatal mice, and the mice were observed for 18–24 hours postinjection (Table 2). When individual NAK mAbs were injected, none of them induced gross blistering; this was expected, as

Dsg1 co-expression in the skin of neonatal mice compensates for the impaired adhesive function of Dsg3 (Figure 3; Amagai, 1999; Mahoney *et al.*, 1999). However, all of the mAbs except NAK3 induced varying degrees of microscopic blistering, with histological evidence of suprabasilar acantholysis (Figure 3). We scored the extent of this blistering using the total area of blisters observed in histology (Table 2).

To overcome the compensation by Dsg1, we used IgG prepared from pemphigus foliaceus (PF) sera that contained anti-Dsg1 IgG autoantibodies. We injected one-half of the minimum dose of PF IgG that had been shown to induce gross blisters, alone or in combination with NAK mAbs (Tsunoda *et al.*, 2003). Neonatal mice that were co-injected with each of the NAK mAbs (except NAK3 and NAK5) developed extensive gross blisters with suprabasilar acantholysis, while mice injected with the PF IgG alone did not show any blister formation (Figure 3).

These findings indicate that most of the NAK mAbs are able to induce the loss of cell–cell adhesion of keratinocytes in neonatal mice. Only two failed to show pathogenic effects.

Table 2. Summary of the pathogenic activities of NAK mAbs in passive transfer assay

	NAK alone[1]			NAK+PF IgG[2]	
			Blister formation		
NAK	*n*	IgG deposition	Histologic blister Scores[3]	*n*	Gross blisters
1	3	+	5.70	3	3
2	3	+	16.31	2	2
3	3	+	0	2	0
4	3	+	38.91	3	1
5	3	+	4.04	2	0
7	3	+	4.82	2	2
8	5	+	6.38	2	2
9	3	+	22.23	2	2
10	3	+	4.77	4	2
11	3	+	9.48	2	2

PF, pemphigus foliaceus.
[1]NAK mAbs alone were subcutaneously injected into neonatal mice and the formation of gross or microscopic blisters was noted.
[2]NAK mAbs were coinjected with 50% of the minimum dose of PF IgG into neonatal mice and the formation of gross blisters was noted. The number of mice with the gross blisters was listed.
[3]The blister score is defined in Materials and Methods.

None of the individual NAK mAbs induce the PV phenotype in adult mice

To further evaluate the pathogenic activities of NAK mAbs, we inoculated individual hybridoma cells intraperitonealy into Rag2$^{-/-}$ immunodeficient mice (6–8 weeks old), and looked for the appearance of the PV phenotype, which includes weight loss, patchy hair loss, and mucosal erosions (Figure 4, Table 3). None of the recipient mice developed this phenotype, even after obvious ascites fluid formation at day 15. Although all the mice showed *in vivo* IgG deposition on keratinocyte cell surfaces of the skin and mucous membranes, no blister formation was apparent in the oral mucosa at the histological level (Figure 4).

These findings indicate that none of the individual NAK clones inoculated was potent enough to induce the loss of cell–cell adhesion of keratinocytes *in vivo*, during ascites formation in adult mice.

Combined NAK mAbs induce the PV phenotype in adult mice

To explore the possibility of combined pathogenic effects, we inoculated NAK hybridoma cells intraperitonealy in various combinations into Rag2$^{-/-}$ mice (Figure 4, Table 3). When hybridoma cells for NAK1, 2, 4, 7, 8, 9, 10, and 11 were combined, the recipient mice developed weight loss, patchy hair loss, and crusted erosions around the snout, at approximately days 10–15 after the inoculation. These mice showed suprabasilar acantholysis of the oral mucosa and in the skin around the snout, together with *in vivo* IgG deposition on keratinocyte surfaces. The titers of circulating anti-Dsg3 IgG in the recipient mice with combined NAK hybridoma cells were compatible with those with single NAK hybridoma cells. The development of the PV phenotype was observed even after mAbs NAK8, NAK4, NAK10, and NAK9 were sequentially removed from the combination (Table 3). The minimum combination that was sufficient to induce the PV phenotype in all recipient mice tested was NAK1, NAK2, NAK7, and NAK11, or NAK2, NAK3, NAK5, and NAK11,

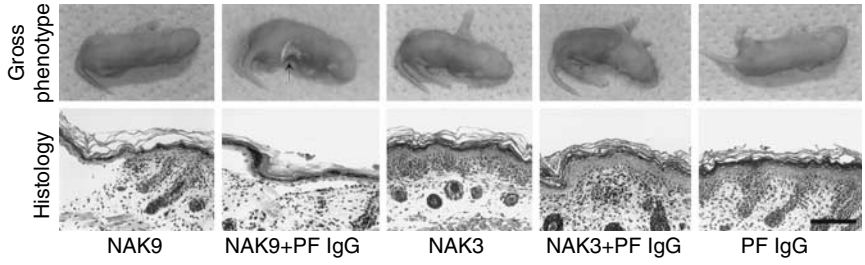

Figure 3. Passive transfer assay of NAK mAbs in neonatal mice. All antibodies except NAK3 and NAK5 mAbs (which recognize Ca^{2+}-independent epitopes) induced gross PV blisters when co-injected with a dose of PF IgG insufficient to induce blisters on its own. Representative data are shown. Neonatal mice injected with NAK9, but not with NAK3, developed microscopic blisters with histological suprabasilar acantholysis, without apparent gross blisters. Neonatal mice co-injected with NAK9, but not NAK3 or PF IgG alone, developed extensive blistering (arrows) with suprabasilar acantholysis. Bar = 50 μm.

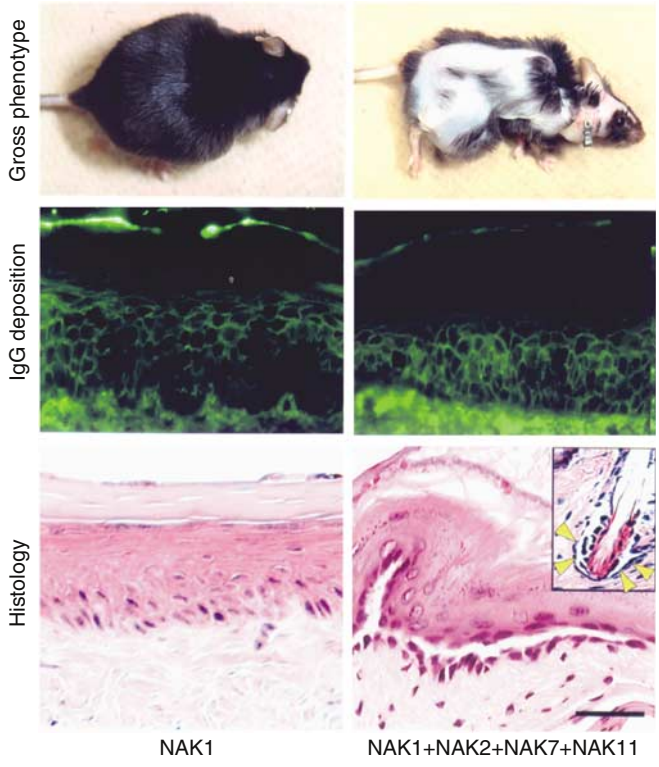

Figure 4. Combinations of NAK mAbs induced the PV phenotype when their hybridoma cells were inoculated intraperitonealy, while the individual mAbs failed to do so. Mice inoculated with NAK1 hybridoma cells alone did not develop apparent PV phenotype (day 15 after inoculation) or histological suprabasilar acantholysis, in spite of apparent ascites formation and *in vivo* IgG deposition on keratinocyte cell surfaces of stratified squamous epithelia. In contrast, when hybridoma cells producing NAK1, NAK2, NAK7, and NAK11 mAbs were combined, recipient mice developed patchy hair loss as well as weight loss (day 15). Histology of the oral mucosa showed *in vivo* IgG deposition and suprabasilar acantholysis and the skin showed acantholysis around telogen hair club (inset; arrow heads indicate the location of acantholysis). Bar = 50 μm.

Table 3. Summary of the pathogenic activities of NAK mAbs in ascites formation assays

NAK	*n*	IgG deposition[1]	PV phenotype[2] (*n* (%))	Titers of circulating anti-Dsg3 IgG[3]
1	7	+	0	144.2±9.3
2	4	+	0	72.9±1.3
3	10	+	0	230.6±38.9
4	5	+	0	ND
5	11	+	0	181.0±20.0
7	3	+	0	92.0±1.0
8	4	+	0	85.3±1.3
9	8	+	0	111.6±4.2
10	8	+	0	41.1±3.3
11	6	+	0	ND
1,2,4,7,8,9,10,11	5	+	5 (100)	114.0±14.7
1,2,4,7,9,10,11	3	+	3 (100)	113.6±0.1
1,2,7,9,11	2	+	2 (100)	ND
1,2,7,11	7	+	7 (100)	126.5±5.9
2,3,5,11	3	+	3 (100)	219.3±5.9
2,7,11	3	+	2 (67)	77.2±6.7
1,2,11	4	+	2 (50)	112.1±2.8
1,2,7	4	+	0	218.3±5.9
1,7,11	4	+	0	175.5±13.0
2,3,5	3	+	0	208.8±5.9
2,11	3	+	1 (33)	55.7±5.6
2,7	2	+	0	94.1±21.5
7,11	3	+	0	ND

ND, not determinded; PV, pemphigus vulgaris.
Hybridoma cells for individual NAK clones or combinations were inoculated intraperitonealy into Rag2$^{-/-}$ mice.
[1]IgG deposition on mouse keratinocyte cell surfaces was observed in all mice injected with NAK mAb hybridomas.
[2]The development of the PV phenotype, including patchy hair loss and suprabasilar acantholysis of the oral mucosa, was evaluated. The number of mice with the PV phenotype was listed.
[3]Titers of circulating anti-Dsg3 IgG were measured by mDsg3 ELISA at days 15–20 days after inoculation.

each of which recognize different epitopes on mouse Dsg3 (Table 1). The mice inoculated with hybridoma cells from these clones developed the full spectrum of PV phenotypic markers (Figure 4).

When we removed any NAK hybridoma cells from the above minimum combination, some of the recipient mice started not to develop the PV phenotype and the other mice showed the PV phenotype at approximately days 15–20 after the inoculation (Table 3). The minimum combination that could induced the PV phenotype at least one of the mice tested was NAK2 and NAK11 hybridoma cells (Table 3).

These findings indicate that NAK mAbs that are not sufficient to induce blisters singly are capable of doing so in adult mice when they are combined.

Pathogenic ranking of NAK mAbs by *in vitro* dissociation assay

The passive transfer assay with neonatal mice and the ascites formation assay with Rag2$^{-/-}$ adult mice require relatively large amounts of antibodies, and do not necessarily simulate pathogenesis in a quantitative fashion. We recently developed an *in vitro* dissociation assay using primary cultures of normal human keratinocytes, as a simple method to quantify the pathogenic strength of pemphigus autoantibodies (Ishii *et al.*, 2005). For this study, we modified the assay to evaluate the pathogenic activity of NAK mAbs against mouse keratinocytes. To obtain Dsg3-dominated cell–cell adhesion, we used primary cultured mouse keratinocytes, which express a low level of Dsg2, in the presence of exfoliative toxin A, which specifically digests Dsg1 (Amagai *et al.*, 2000a). After incubation with various NAK mAbs, a sheet of keratinocytes, released by dispase, were subjected to mechanical stress by pipetting, and the number of cell sheet fragments was determined. The number of cell fragments

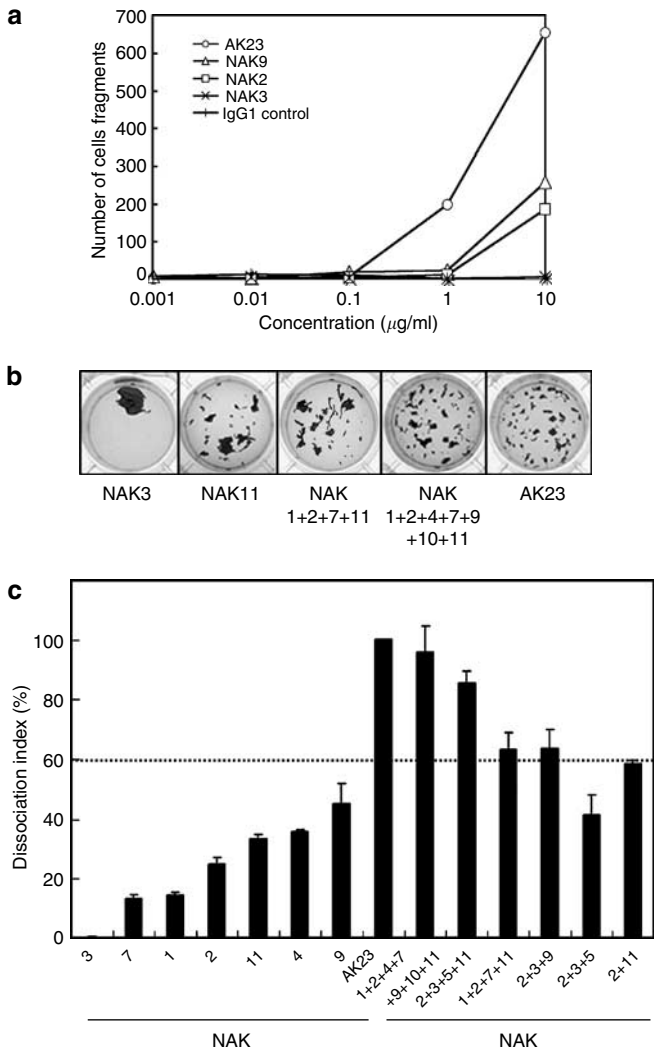

Figure 5. *In vitro* keratinocyte dissociation assays demonstrated synergistic pathogenic effects of NAK mAbs in the loss of keratinocyte cell–cell adhesion. Sheets of mouse keratinocytes were treated with exfoliative toxin A to digest Dsg1and with NAK mAbs, and subjected to mechanical stress. Cell fragments were counted by image analysis. Representative data are shown. (**a**) Dose dependency of NAK mAbs on dissociation scores. (**b**) NAK11 induced fragmentation while NAK3 did not. The combined mAbs generated similar numbers of cell fragments as did AK23, an anti-Dsg3 mAb that is able to induce the PV phenotype (Tsunoda *et al.*, 2003). (**c**) Dissociation indexes of NAK mAbs alone and in combination. The dissociation index varied among the mAbs. Combinations of mAbs (adjusted to 1 μg/ml) had higher dissociation indexes than individual NAK mAbs. Combining these data with the results from ascites formation assays indicates that the threshold for blister formation *in vivo* may be at a dissociation index of approximately 60% (dashed line).

increased as the concentration of AK23 or various NAK mAbs increased, indicating that this assay evaluate pathogenic strength in a quantitative manner (Figure 5a). Dissociation index values were calculated as a percent of the number of fragments obtained with a positive control mAb, AK23 (Tsunoda *et al.*, 2003).

Each NAK mAb had a different dissociation index, indicating that this assay could discriminate differences in activity among them (Table 1, representative data are shown in Figure 5b and c). NAK3 and NAK5, which failed to induce apparent pathogenic activity in the passive transfer model, gave smaller dissociation indexes than the others (0.5 and 12.5%, respectively), while NAK9 had the highest dissociation index (45.1%).

When several NAK mAbs were combined, without changing the total amount of IgG added (1 μg/ml), dissociation scores greatly increased (Figure 5c). When NAK1, 2, 4, 7, 9, 10, and 11 mAbs were combined, the dissociation score was equivalent to that of the AK23 mAb, hybridoma cells of which can alone induce the PV phenotype in adult mice (Tsunoda *et al.*, 2003). When we removed some of NAK mAbs from the combination, the dissociation scores gradually decreased in general. The dissociation scores of the all combinations were still higher than those of the individual NAK mAbs, indicating a synergistic pathogenic effect (representative data in Figure 5c). Considering these results with those of the ascites formation assay, we speculate that there may be a threshold of blister formation *in vivo* in adult mice corresponding to a dissociation index of approximately 60% (dashed line in Figure 5c).

These findings indicate that individual NAK mAbs have various pathogenic activities in disrupting the cell–cell adhesion mediated by Dsg3, none individually potent enough to induce an apparent PV phenotype in adult mice. However, combinations of these mAbs show synergistic effects above the threshold for blister formation and development of the PV phenotype.

DISCUSSION

In this study, we generated 10 anti-Dsg3 IgG mAbs from PV model mice produced by adoptive transfer of naive Dsg3$^{-/-}$ splenocytes. All mAbs specifically reacted with native mouse Dsg3, but not with mouse Dsg1. Some (NAK1, 3, 4, 7, 8, 9) cross-reacted with human Dsgs, although the isoform specificities of the mAbs were not necessarily conserved between mouse and human. NAK1, NAK3, and NAK9 reacted only with human Dsg3, while NAK4 reacted with both human Dsg3 and Dsg1, and NAK7 and NAK8 reacted only with human Dsg1. These findings indicate that antibodies against Dsgs have fine specificities, determined by the few amino-acid residues that are not conserved between mouse and human isoforms. This highlights the difficulty of performing detailed evaluations of these mouse antibodies using human skin or keratinocytes and *vice versa*.

We performed three different assays to evaluate the pathogenic activities of NAK mAbs: passive transfer, ascites formation, and *in vitro* dissociation. The passive transfer assay, by injection of IgG into neonatal mice, is well established (Anhalt *et al.*, 1982), and the ascites formation assay, after inoculation of hybridoma cells into immunodeficient adult mice, has been developed to evaluate *in vivo* pathogenic activity of anti-Dsg3 mAbs (Tsunoda *et al.*, 2003). The passive transfer assay is more easily controlled than the ascites formation assay. Highly concentrated IgG can be applied to neonatal mice by passive transfer, while the

amount of IgG in an ascites formation assay is dependent on the production rate of the hybridoma cells. Although these two assays reflect *in vivo* pathogenic activity of anti-Dsg3 IgG and subjective scaling can be used to represent the extent of the phenotypic results (Mahoney *et al.*, 1999; Aoki-Ota *et al.*, 2004), these assays do not measure pathogenic strength in a quantitative manner. To compare pathogenic strengths among pemphigus sera or anti-Dsg3 IgG mAbs, an *in vitro* dissociation assay was developed using primary cultures of human keratinocytes (Ishii *et al.*, 2005). In this study, we modified that assay using mouse keratinocytes to analyze the potential of each NAK mAb.

Under passive transfer, eight of 10 mAbs showed pathogenic activity and induced blister formation (Figure 3, Tables 1 and 2), while only two of eight AK mAbs, isolated from PV model mice receiving immunized Dsg3$^{-/-}$ splenocytes, were pathogenic (Tsunoda *et al.*, 2003). The higher frequency of pathogenicity among NAK mAbs is consistent with the fact that although mice receiving naive Dsg3$^{-/-}$ splenocytes showed lower titers of anti-Dsg3 IgG than did those receiving immunized splenocytes, the two types of mice showed no apparent differences in disease severity (Aoki-Ota *et al.*, 2004). The eight pathogenic NAK mAbs recognized Ca^{2+}-dependent epitopes on Dsg3, while the epitopes of the two nonpathogenic NAK mAbs were not dependent on Ca^{2+} (Table 1). Similar results have been seen with the AK mAbs: the two pathogenic antibodies were dependent on Ca^{2+}, while the six nonpathogenic AK mAbs were independent of Ca^{2+} (Tsunoda *et al.*, 2003). Most of pemphigus patients' IgG autoantibodies recognize Ca^{2+}-dependent epitopes on Dsg3 (Matis *et al.*, 1987; Eyre and Stanley, 1988; Amagai *et al.*, 1995). These findings together strongly suggest that pathogenic antibodies recognize Ca^{2+}-dependent conformational epitopes while nonpathogenic antibodies react with Ca^{2+}-independent linear epitopes.

Although eight of 10 NAK mAbs showed pathogenic activity in the passive transfer assay, none induced the PV phenotype in the ascites formation assay (Figure 4, Table 1), indicating that none were as potent as mAb AK23, which could induce the PV phenotype in adult mice upon hybridoma inoculation (Tsunoda *et al.*, 2003). More sensitive in vitro dissociation assays confirmed this and allowed us to rank the pathogenicity of NAK mAbs (Figure 5). In fine mapping using human Dsg1/Dsg3 point-mutated molecules, the epitopes of NAK 1 and 9, which crossreacted with human Dsg3, were mapped, respectively, to the amino-terminal T25, Y28, and Q29 and to T25, Y28, Q29, V53-N56 of Dsg3. These residues form a part of the adhesive interface of Dsg3 as predicted by crystal structure (Boggon *et al.*, 2002), but are different from the AK23 epitope (V3, K7, P8, and D59) (Tsunoda *et al.*, 2003). These findings suggest that the residues V3, K7, P8, and D59 may form the critically important part of the adhesive interface of Dsg3.

Although our attempt to isolate pathogenic anti-Dsg3 mAbs as potent as AK23 was not successful, pemphigus patients' sera always contain polyclonal anti-Dsg3 IgG autoantibodies, which recognize different parts of the molecule (Amagai *et al.*, 1992; Sekiguchi *et al.*, 2001). We therefore tested the possibility that a combination of several weakly pathogenic mAbs might show a synergistic effect and induce the PV phenotype in adult mice. Indeed, when the combination of hybridoma cells producing NAK1, 2, 4, and 7–11 were inoculated into Rag2$^{-/-}$ mice, the recipients developed the PV phenotype, with weight loss, patchy hair loss, and crusted erosions around the snout (Table 3). The minimal combination tested that was sufficient to induce the phenotype in all mice tested was NAK1, 2, 7, and 11 mAbs or NAK2, 3, 5, 11 mAbs (Figure 4). *In vitro* dissociation assays confirmed the synergistic pathogenic effects of NAK mAbs (Figure 5). This is the first demonstration of synergism among antidesmoglein IgG antibodies in inducing the loss of cell–cell adhesion of keratinocytes *in vivo* and *in vitro*.

In a previous study, AK mAbs recognizing the middle to C-terminal extracellular domain of Dsg3 did not show apparent pathogenic activity, suggesting that IgGs reacting with functionally less important parts of the molecule do not contribute to pathogenesis (Tsunoda *et al.*, 2003). However, in this study, NAK mAbs recognizing such regions showed synergistic effects with mAbs reacting with the N-terminal domain (e.g., mAbs NAK2, 3, and 9 or NAK2 and 11 in Figure 5c, Table 3). These findings provide molecular evidence that anti-desmoglein IgG antibodies recognizing the middle to C-terminal extracellular domains of Dsg3 can also take part in the pathogenic process of blister formation.

We knew that the dose of antibodies is an important factor to determine the pathogenic strength of patients' sera because antibody titers are generally correlated with the disease activities when monitored in individual patients (Sams and Jordon, 1971; Ishii *et al.*, 1997; Cheng *et al.*, 2002). We also knew that the epitope of antibodies is another important factor to determine the pathogenic strength of each anti-Dsg3 IgG (Tsunoda *et al.*, 2003). In this study, we demonstrated that the combination of different anti-Dsg3 IgG autoantibodies is the third important factor because most, if not all, patients' sera contain polyclonal anti-Dsg3 IgG. Thus, the clinical severity of the disease in each patient may be determined in a more complex manner than initially thought.

The preclinical condition in patients that precedes development of PV remains to be elucidated. One possibility is that one or a few nonpathogenic or weakly pathogenic anti-desmoglein IgG autoantibody (or autoantibodies) are first generated without inducing the apparent disease; additional autoantibodies recognizing different epitopes on desmoglein are subsequently produced, with the onset of the disease phenotype. It would be interesting to study whether signal transduction induced by the combined weakly pathogenic NAK mAbs and by the single AK23 mAb are different. Also of interest is whether long-term *in vivo* binding of weakly pathogenic antibodies induces any secondary inflammation in mice, including the recruitment of eosinophils. The weakly pathogenic anti-Dsg3 NAK mAbs together with the potent AK23 mAbs provide valuable tools to replicate and study the polyclonal condition as found in patients, and to dissect the molecular pathophysiology of pemphigus.

MATERIALS AND METHODS

Mice

Dsg3$^{-/-}$ offspring were obtained from matings of homozygotes (Amagai *et al.*, 2000b; Tsunoda *et al.*, 2002). These mice had the mixed genetic background of 129/SV (H-2^{b}) and C57BL/6J (H-2^{b}). Splenocytes (5×10^{7}) were prepared from naive Dsg3$^{-/-}$ mice, and adoptively transferred into C57BL/6 Rag2$^{-/-}$ mice (Central Institute for Experimental Animals, Tokyo, Japan) via the tail vein, as previously described (Aoki-Ota *et al.*, 2004). Anti-Dsg3 antibody production was examined by ELISA for recombinant mouse Dsg3, and *in vivo* binding against Dsg3 was confirmed by live keratinocyte staining (Tsunoda *et al.*, 2002). All mouse studies were approved by the Animal Ethics Review Board of Keio University.

Production of NAK mAbs

Splenocytes were isolated from mice that had the active PV phenotype and fused with P3 mouse myeloma cells at a ratio of 5:1 with PEG 4000 (Merck, Darmstadt, Germany), followed by selection with hypoxanthine–aminopterin–thymidine in the presence of 10% hybridoma cloning factor (IGEN, Gaithersburg, MD). Hybridoma cells were screened by ELISA using recombinant mouse Dsg3 and positive clones were further examined by live keratinocyte staining. Ten positive clones were screened and designated as NAK (AK mAb from PV model mice by naive splenocyte transfer) (Table 1). Each clone was obtained by three replicates of the limiting dilution method. The isotypes of mAbs produced were determined using an isotyping kit (Roche Diagnostics, Mannheim, Germany). Antibodies were purified from culture supernatants using the HiTrap rProtein A FF column (Amersham Biosciences, Buckinghamshire, UK).

ELISA and live keratinocyte staining

The reactivities of antibodies with mouse Dsg3, mouse Dsg1, human Dsg3, and human Dsg1 were measured by ELISA using the respective recombinant Dsg molecules, as described previously (Amagai *et al.*, 2000b; Tsunoda *et al.*, 2003; Anzai *et al.*, 2004). In brief, each sample was diluted 50,000-fold and run in duplicate. A standard serum obtained from a Dsg3$^{-/-}$ mouse immunized with mDsg3$^{-/-}$ was used as a positive control, and serum from a nonimmunized mouse was used as a negative control. The ELISA scores were calculated as index values using the formula: index value = (OD_{450} of sample − OD_{450} of negative control)/(OD_{450} of positive control − OD_{450} of negative control) × 100. Live keratinocyte staining was performed using the mouse keratinocyte cell line PAM212, as described previously (Amagai *et al.*, 2000b; Tsunoda *et al.*, 2003). To confirm calcium sensitivity of epitopes, an EDTA-treated ELISA was performed, incubating the ELISA plate with 5mM EDTA prior to the assay.

Epitope mapping by immunoprecipitation using chimeric desmogleins

Immunoprecipitation was performed using domain-swapped or point-mutated Dsg1/Dsg3 molecules to determine mAb epitopes (Tsunoda *et al.*, 2003; Anzai *et al.*, 2004). Typically, 3 μg of purified mAb and 200 μl of culture supernatant containing recombinant protein were mixed and incubated at room temperature for 30 minutes. These proteins were immunoprecipitated with protein G-Sepharose (Amersham Biosciences) overnight at 4°C. Anti-E-tag mAb (Amersham Biosciences) was used as a positive control. The immunoprecipitates were fractionated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, MA). The recombinant proteins were visualized with an anti-6x histidine Ab (R&D Systems, Minneapolis, MN).

Passive transfer assays using neonatal mice

The pathogenic activities of the mAbs were evaluated by passive transfer in neonatal mice, an established assay for the pathogenic activity of pemphigus sera (Anhalt *et al.*, 1982; Amagai *et al.*, 1994). We injected NAK mAbs (120–250 μg/mouse, the highest dose we injected per mice) either alone or together with a small amount of total IgG prepared by ammonium sulfate precipitation of PF serum. The dosage of PF IgG was set at 50% of that needed to induce gross blistering, that is, 1 mg/mouse. Neonatal ICR (Institute of Cancer Research) mice (Sankyo Lab Service, Tokyo, Japan) were used at 12–24 hours after birth (body weight 1.5–2.0 g). The skin was evaluated grossly and microscopically 18–24 hours after injection. To evaluate microscopic blisters, the entire body skin was sectioned into six strips of about 3 mm in width. Blister formation was considered positive when the length of a single lesion was greater than 120 μm or when more than two acantholytic lesions were found after examining all six sections. Blisters found at the edges of sections were not counted. To quantify the extent of blister formation histologically, we calculated scores using the following formula: histologic blister score = ((combined total length of all blistered regions)/(combined total length of all sections examined)) × 100.

Ascites formation assay

To evaluate the pathogenic activity of the NAK mAbs in adult mice, we inoculated 5×10^{6}–1×10^{7} cells, from individual hybridomas or from a mixture of clones, into the peritoneum of Rag2$^{-/-}$ mice primed with 2,6,10,14-tetramethyl-pentadecane (Wako Pure Chemical Industries, Osaka, Japan). The inoculated mice were monitored for ascites formation as well as for the appearance of the PV phenotype, manifested by weight loss and patchy hair loss. Biopsies of the oral mucous membranes and skin were taken when mice developed the PV phenotype or when ascites formation was observed after day 14.

In vitro dissociation assay

To evaluate the activity of mAbs in the inhibition of keratinocyte cell–cell adhesion, we modified a previously described *in vitro* dissociation assay (Ishii *et al.*, 2005). For this study, we used primary cultures of mouse keratinocytes rather than human keratinocytes. For these cultures, skin specimens prepared from neonatal ICR mice at 12–24 hours of age were incubated in dispase II (Roche Diagnostics Corp., Mannheim, Germany), and separated epidermis samples were further incubated in 0.25% Trypsin (Invitrogen, Carlsbad, CA). Isolated keratinocytes were dispensed into 12-well culture plates with keratinocyte culture medium CnT-02 (CellnTec, St Gallen, Switzerland). When keratinocytes were confluent, 1.2 mM calcium was added and cells were incubated for 24 hours. Recombinant exfoliative toxin A (0.25 μg/ml) produced in *E. coli*, which specifically digests mouse Dsg1 (Amagai *et al.*, 2000a; Amagai *et al.*, 2002), was added to cultures 2 hours before the assay, in which 1 μg/ml of individual or pooled NAK mAbs (1 μg/ml total) were added to culture media. After washing with 0.9 mM Ca^{2+}-phosphate-buffered saline

twice, mouse keratinocytes were incubated with dispase II for 15 minutes to release cells as sheets. Released sheets were carefully washed twice with 0.9 mM Ca^{2+}-phosphate-buffered saline and subjected to mechanical stress by pipetting with a 1 ml disposable pipette tip. Fragments were fixed by adding formaldehyde to a final concentration of 3% and were stained with crystal violet (Sigma-Aldrich Co., St Louis, MO). A cell sheet treated with 1 μg/ml of the positive control mAb AK23 was included in each assay to adjust for inter-assay variability. The mean number of particles was determined by counting with Image Pro software (Media Cybernetics Inc., Silver Spring, MD), using three sets of digital images captured for each plate. Dissociation scores were calculated from the number of fragments (N) as follows: Dissociation score $= ((N$ with mAb$-N$ without mAb$)/(N$ with AK23$-N$ without mAb$)) \times 100$.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Figure 1. Immunostaining of mouse and human tissues with NAK mAbs that were not listed in Figure 1.

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